An investigation into the adaptive response to MNNG of chinese hamster cells in vitro

McDowall, Gordon David

Award date:
1988

Awarding institution:
University of Bath

AN INVESTIGATION INTO THE ADAPTIVE RESPONSE TO MMNG OF CHINESE HAMSTER CELLS *IN VITRO*.

THESIS.

Submitted by Gordon David McDowall, B.Sc., for
the degree of Doctor of Philosophy
of the University of Bath
1988

This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of C.J. Soper, B.Pharm., M.Sc. Ph.D., M.P.S.

SIGNED: Gordon McDowall.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor, Dr. C.J. Soper for his invaluable guidance and encouragement throughout this project. I am also indebted to many members of the academic staff for their assistance particularly Dr. G.H. Dewar and Dr. T.M Jefferies for their helpful advice.

Sincere thanks are offered to the technical staff in the School of Pharmacy, all of whom provided excellent technical assistance at some stage during this work.

I would also like to thank the staff of the Regional Cytogenetics Unit, Southmead, Bristol for their help with the SCE work.

Finally, I would like to thank the Post-graduate students of the School of Pharmacy and Pharmacology, past and present, for preserving my sanity and making my time spent within this School an interesting and enjoyable one.

This work was supported by a University of Bath Research Award.

This Thesis is dedicated to my Mum and Dad, for their unfailing support.

Yesterday is already a dream,

And tomorrow is only a vision;

But today well lived, makes every

Yesterday a dream of happiness,

And every tomorrow - a vision of hope.

Sanskrit.

ABBREVIATIONS

AAF - 2-acetylaminofluorine
BCNU - 1,3-bis(2-chloroethyl)-1-nitrosourea
BrdU - 5-bromodeoxyuridine
CCNU - 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
DMN - dimethylnitrosamine
DMSO - dimethylsulphoxide
EMS - ethylmethanesulphonate
ENU - ethylnitrosourea
FCS - foetal calf serum
HCHO - formaldehyde
HGPRT - hypoxanthine-guanine-phosphoribosyl-transferase (E.C. 2.4.2.8.)
Hoechst 33258 -(2-(-2-4-hydroxyphenyl)-6-benzimidazolyl)-6
-(1-methyl-4-piperazyl)-benzamidazole 3HCl
HPLC - high performance liquid chromatography
3MA - 3-methyladenine
3MG - 3-methylguanine
7MG - 7-methylguanine
3-MAG - 3-methyladenine-DNA-glycosylase II
Me-CCNU - 1-methyl-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
Mer $^{-}/^{+}$ - methyl exision repair deficient/proficient
MMS - methylmethanesulphonate
MNNG - N-methyl-N'-nitro-N-nitrosoguanidine
MNU - N-methylnitrosourea
Na+/K+ATPase - sodium-potassium-magnesium-dependent adenosine
triphosphatase (E.C. 3.6.1.3.)
NMR - nuclear magnetic resonance
NNK - 4-(N-methyl-nitrosamino)-1-(3-pyridyl)-1-butanone
NNN - nitrosonornicotine
ODS - octadecylsilane
O^6MG - O^6-methylguanine
O^6MGMT - O^6-methylguanine-DNA-methyltransferase
Oua - ouabain
SCE - sister chromatid exchange(s)
SCX - strong cation exchange
TG - thioguanine
TMS - tetramethylsilane
UV - ultraviolet light

SUMMARY

The introduction to this thesis discusses the occurrence and potential carcinogenic hazards of environmental N-nitroso compounds. The adaptive response to MNNG, which arises from the chronic administration of this N-nitroso compound, is discussed with reference to the current knowledge of this response in *E.coli*, mammals and mammalian cells *in vitro*. Chapter 2 lists the materials and methods basic to the cell culture procedures used in succeeding chapters.

The experimental work is presented in five parts. In Chapter 3, the establishment of the adaptive pre-treatment protocol used throughout this study is described.

Chapter 4 describes the effect of adaptive pre-treatment on the growth, MNNG-induced cytoxicity and mutation to ouabain resistance (Oua^R) in CHO-K1 and V79-379A cells. The effect of cell synchrony on the MNNG-induced cytotoxicity and mutation to Oua^R in CHO-K1 cells is also described.

In Chapter 5 the isolation of cell clones that are either resistant or sensitive to the cytotoxic action of MNNG, is described. The growth parameters, MNNG-induced cytotoxicity and mutation to Oua^R of each clone are determined, and compared to those found for adaptively pre-treated clones and their respective parental cells.

Chapter 6 describes the ability of cell-free extracts of CHO-K1, V79-379A cells, and their MNNG-resistant clones, to de-methylate O^6-methylguanine within an alkylated DNA substrate. The assay system has been validated using mouse C3H $10T^1/_2$ cells, a cell line previously shown to de-methylate alkylated DNA.

Chapter 7 describes the detection of MNNG-induced sister chromatid exchanges (SCE) in CHO-K1, V79-379A and their respective MNNG-resistant clones, and investigates the effect of adaptive pre-treatment on this parameter.

A summary of findings is presented at the end of each experimental chapter. These are discussed in Chapter 8 in the light of current evidence for the existence of an adaptive response in mammalian cells.

CONTENTS

CHAPTER 1. INTRODUCTION.

1.1. Environmental N-nitrosocompounds.

Interest in environmental chemical carcinogenesis probably began in 1775 when the London surgeon Sir Percival Pott published a treatise entitiled "Chirurgical Observations Relative to the Cateracts, the Polyps of the Nose, the Cancer of the Scrotum, the Different Kinds of Ruptures and the Mortification of the Toes and Feet", in which he concluded that the high incidence of epithelioma of the scrotum among chimney sweeps, was due to the soot to which they were daily exposed. He wrote, "It is a disease which always makes its first attack on, and its final appearance in the inferior part of the scrotum; where it produces a superficial, painful, ragged, ill-looking sore with hard and rising edges. The trade call it soot-wart....the disease, in these people, seems to derive its origin from a lodgement of soot in the rugae of the scrotum". Sir Potts' observations are historically acknowledged as having provided the first evidence for a role of environmental agents in the aetiology of cancer. They also provided the basis for the first preventative measures against environmental cancer when, in 1778, the Danish chimney-sweeps' guild urged its members to bathe daily (Clemmensen, 1951), and by 1892 a lower scrotal cancer incidence in northern European chimney sweeps, relative to that in England, was noted (Butlin, 1892). The same disease was once again noticed among workers in the Lancashire cotton spinning industry where it became known as "mule spinners cancer". About this time, occupational skin cancer among oil and tar workers was reported by Von Volkemann, (1875) in Germany and by Bell, (1876) in Scotland. It was suspected that prolonged contact of the spinners with clothes impregnated with

mineral oil used to lubricate their machines was responsible for the disease. Some years later, a very potent carcinogenic polycyclic hydrocarbon called benz(a)pyrene was identified and isolated from crude coal tar (Kennaway, 1955).

Many known carcinogens interact with various components of DNA, and in many cases cause damage to the nitrogenous bases. Some chemical carcinogens, such as alkylating agents, are reactive with DNA in the native state and do not require prior chemical modification in target cells (Singer, 1979). Others, such as benz(a)pyrene, are essentially unreactive with DNA. It is known, however that benz(a)pyrene, and other polycyclic aromatic hydrocarbons are metabolised to phenols and dihydrodiols by the mammalian P-450 system (Selkirk *et al.*, 1982). Some of the products of this metabolism are electrophilic epoxides and it is well established that the ultimate carcinogenic form of benz(a)pyrene is an anti diol epoxide which can react with the 2-amino group of guanine, amongst other sites (Malaveille *et al.*, 1977; Weinstein *et al.*, 1976). Thus it was shown that metabolic activation can convert relatively weak *proximal* carcinogens into more potent *ultimate* carcinogens.

The earliest impetus for the study of the interaction of chemicals with DNA was probably their potential for use as lethal and injurous agents in warfare, but a far more humane stimulus came from the field of cancer chemotherapy. This was based on the simple idea that damage to DNA can interfere with normal DNA synthesis, leading to the replicative arrest of rapidly dividing cell populations such as cancer cells. Increasing public awareness of environmental mutagens and carcinogens has, in recent years, led to an interest in the study of the mechanisms by which

genotoxic chemicals interact with and damage DNA.

The human environment constantly presents threats to our genetic material and if it were not for continuous cellular monitoring and repair systems, we would soon be living on a sick and barren planet. The study of DNA repair is therefore very important in the search for a fuller understanding of the mechanisms of human carcinogenesis. One of the most persuasive arguments for a role of DNA damage and mutation in the pathogenesis of at least one human cancer, stems from the study of patients suffering from the hereditary disease Xeroderma pigmentosum (XP). Extremely high incidences of skin cancer were recorded in patients with XP, and cells isolated from patients by Cleaver in 1968, revealed a defect in the excision repair of base damage caused by UV irradiation. Skin cancers occuring in patients with this disease are probably caused by UV radiation from the sun. This was the first indication of a DNA repair defect associated with a a human disease. Cleavers' observations also provided an impetus to examine the phenotypic resonses to DNA-damaging agents of cells from a number of other hereditary human diseases, particularly those associated with chromosomal abnormalities or with an abnormally raised incidence of neoplasia (Arlett and Lehman, 1978; Lehman and Mayne, 1981; Paterson, 1979; Setlow, 1978).

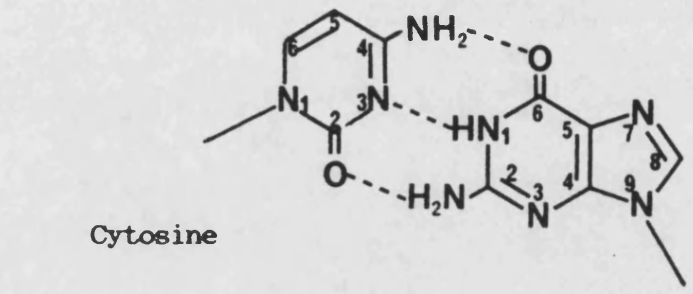
The past few decades have witnessed an unparalleled expansion of the chemical industry with the concomitant development of many new organic chemical products as well as enhanced product application e.g. increased usage of agricultural pesticides and fertilizers. It is estimated that over 3000 chemicals are now known to be carcinogenic in experimental animals

and it seems likely that this number will increase since approximately 700 to 3000 *new* industrial chemicals are introduced *per annum* into the United States alone (Fishbein, 1979). The interest and concern of the general public has understandably increased as the list of known environmental carcinogens expands.

Of the many thousands of chemicals known to man, the alkylating agents probably represent one of the major classes of DNA-damaging agents to which the human species is exposed (Fishbein, 1979). They were among the first chemicals to be recognised as mutagenic (Auerbach and Robson, 1946). Alkylating agents are electrophilic compounds with affinity for nuceophilic centres in organic macromolecules (Lawley, 1974; Singer and Kusmierek, 1982). They can be either monofunctional or bi- or polyfunctional, the former having a single reactive group which can covalently interact with single nucleophilic centres in DNA. The bifunctional alkylating agents have two reactive groups allowing them to react with molecules either within the same strand of DNA (intra-strand cross-links), or with molecules on different DNA strands (inter-strand cross-links). Numerous sites for alkylation have been identified in all four bases, although not all of them have equal reactivity (1.4.2.). Figure 1.1. shows normal purine:pyrimidine base pairing and also the sites, within these molecules, susceptible to alkylation by alkylating agents. In general the ring nitrogens of the bases are more nucleophilic than the oxygens, with the N^7 position of guanine and N^3 position of adenine being the most reactive, (Roberts, 1978). Alkylation of oxygen in phosphodiester linkages results in the formation of phosphotriesters.

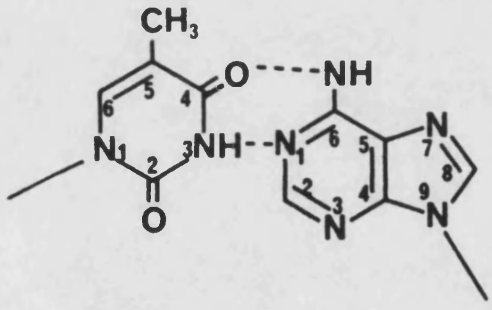
Alkylating agents of particular importance are the

Figure 1.1. Normal complementary DNA base pairing



Cytosine

Guanine



Thymine

Adenine

Sites susceptible to methylation in DNA and RNA.

Guanine	N^1, N^2, N^3, N^7, O^6.
Adenine	N^1, N^3, N^6, N^7.
Cytosine	N^3, N^4, O^2.
Thymine	O^2, O^4, N^3.
Ribose	2'-OH
Phosphate	O.

N-nitroso compounds e.g. nitrosamines and nitrosamides, since they possess the property of generating cancer in a wide range of animal species. Other compounds possess this ability but no single chemical group is as universally carcinogenic as the N-nitroso compounds. In a number of studies, about 300 different N-nitroso compounds were administered to more than 40 different vertebrate species. Almost 90% of the tested compounds induced tumours in one species or another (Lijinsky, 1984; Preussman and Stewart, 1984). These observations may suggest that humans are also susceptible to carcinogenesis by N-nitroso compounds and that exposure entails a probable carcinogenic risk.

N-nitroso compounds are formed readily by interaction of a secondary or tertiary amino compound with a nitrosating agent. Common nitrosating agents include inorganic nitrites, nitrite and nitrate esters, nitrogen oxides and other N-nitroso compounds. Nitrosamines are produced by the reaction of secondary amines with nitrous acid giving a characteristic yellow oil. This feature is sometimes used as an analytical test to establish the presence of secondary amines in a mixture of primary, secondary and tertiary amines.

$$R_2NH + HNO_2 \xrightarrow{HCl} R_2N\text{-}N{=}O + H_2O$$

2° amine — nitrous acid — nitrosamine

These compounds are found extensively in certain industrial environments, in particular the rubber, leather and agricultural industries. Di-methylnitrosamine (DMN) $(CH_3)_2N\text{-}NO$, was once used as an industrial solvent but the observation that the incidence of liver cirrhosis in exposed workers was higher than other workers,

prompted an investigative study by Magee and Barnes, (1956). Their animal experiments showed the induction of liver necrosis in rats, rabbits, mice and guinea pigs following a single dose of 15 to 50 $mgkg^{-1}$ body weight. Chronic dietary administration of DMN to rats at levels of 100 and 200 parts per million (ppm) led to the death by liver damage within 95 and 37 days, respectively. Levels of 50ppm induced no visible liver damage after 110 days, but following continued treatment every rat developed liver cancer and subsequently died.

N-nitroso compounds are found in foodstuffs containing amines and nitrosating agents which have reacted together. One class of nitrosating agents, commonly found in the environment, are the nitrites. Readily available sources of nitrite occur in the form of sodium and potassium nitrate, widely used as preservatives in cured meat and fish, bacon, cheese etc. Nitrates are reduced to nitrite by bacteria present in the human body e.g. nitrate reducing bacteria in the saliva (Tannenbaum *et al.*, 1977). The cooking process can catalyse the reduction of nitrates to nitrites allowing reaction with amines to form nitrosamines. Trace amounts of nitrosamines have been detected in samples of cooked foods e.g. levels of up to 100 μgkg^{-1} of DMN have been observed consistently in fried bacon (Scanlan, 1975). Injested amines may also react with residual nitrites from cured meat or fish, to form nitroso compounds in the stomach and bladder. The number of N-nitroso compounds which can be formed *in vivo*, from these sources, is very large, and the carcinogenic effects of the majority are still unknown (Lijinsky, 1980).

The smoking of tobacco probably represents one of the principal human exposures to N-nitroso compounds. It was proposed

that amines contained in tobacco smoke, once swallowed, may be nitrosated in the stomach to form carcinogenic nitrosamines whose target might be the lung, amongst other organs (Taylor and Lijinsky, 1975). The identification of DMN in cigarette smoke in 1972, confirmed the suspicions that burnt tobacco itself contained nitrosamines (Rhodes and Johnson, 1972). The studies of Hecht *et al.*, (1978) led to the identification of a number of nitrosamines present in tobacco smoke e.g. nitrosonornicotine (NNN), a product expected from the nitrosation of the tertiary amine nicotine, and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) an open ring form of NNN.

Cosmetics and toiletries contain nitrosamines such as di-ethanolnitrosamine a component of cosmetic emulsifiers, and methylethanenitrosamines which eventually become incorporated into shampoos. In these forms both compounds may be absorbed through the skin (Hecht *et al.*, 1980). Table 1.1. gives examples and sources of N-nitroso compounds commonly encountered by man.

The largest group of amines which are of concern to the population at large, consists of drugs and medicines. Many hundreds of these are secondary or tertiary amines, although only a limited number are in common use. They include tranquilisers, analgesics and antihistamines, some of which are taken in relatively large quantities by many people, for long periods. Cimetidine (Tagamet), a widely used and very effective drug for the treatment of gastric disorders, has been shown to induce stomach tumours after becoming nitrosated *in vivo* (Jensen and Magee, 1981). Many drugs have more than one tertiary nitrogen susceptible to nitrosation, and the potential products are numerous. The extent to which the products contribute to an

Table 1.1. Examples of N-nitroso compounds found in the human environment.

Compound	Source
Di-ethanolnitrosamine	Cosmetics, workplace, Tobacco.
Di-methylnitrosamine	Food, Workplace, Tobacco.
Nitrosonornicotine	Tobacco.
Nitrosomorpholine	Workplace, Tobacco.
Nitrosopyrrolidine	Food, Tobacco.
Nitrosopiperidine	Food, Workplace.
Di-ethylnitrosamine	Food, Workplace
Methyldodecylnitrosamine	Cosmetics.
Methyltetradecylnitrosamine	Cosmetics.
Di-butylnitrosamine	Workplace.
Di-phenylnitrosamine	Workplace.

increased cancer risk, associated with use of the drug, is difficult to calculate, even though one or all of the products have been found carcinogenic in animals (Mergens *et al.*, 1979).

In 1960 considerable interest in nitrosamides was stimulated by the finding that N-methyl-N'-nitro-N -nitrosoguanidine (MNNG) had a reproducible anti-tumour activity against the murine L1210 leukaemia cell line (Green and Greenberg, 1960). In later, structure-activity studies it was demonstrated that the nitroso group of the MNNG molecule was required for its anti-tumour activity and that substitution of a chloroethyl group for the methyl group enhanced its activity (Hyde *et al.*, 1962). Montgomery *et al.*, (1975), evaluated a series of compounds with structural similarities to MNNG, assessing their anti-tumour activities. One of these, N-methyl-N-nitrosourea (MNU), was found to be a very effective anti-tumour agent. This, coupled with the fact that MNU crosses the blood-brain barrier in cytotoxic concentrations, led to the synthesis of a large number of nitrosoureas with the aim of producing a clinically effective cytotoxic agent. Of the 200 or so nitrosourea analogues synthesised by Montgomery, only one specific structural type, i.e. N-(2-haloethyl)-N-nitrosourea, proved to be superior in its anti-tumour action to MNU. Further alterations to the 2-chloroethyl and the nitrogen group led to the synthesis of carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)), lomustine (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)) and its methylated derivative semustine (Me-CCNU), compounds used extensively as anti-neoplastic agents in cancer chemotherapy (Meyn and Murray, 1984). However, as one would expect with N-nitroso compounds, there are numerous reports of secondary malignancies,

particularly leukaemias, lymphomas and squamous cell carcinomas occuring after a 4 year latent period following the initiation of cancer chemotherapy which has included these drugs (Sieber and Adamson, 1975). It has been reported that over a period of 10 years, 5 to 15% of patients given alkylating agents, will develop a secondary cancer of some description (Althouse, 1979).

The number and variety of environmental amines available for nitrosation is enormous. Human contact with such chemicals is likely to be chronic i.e. of the long-period, low-dose type, rather than an acute, single high dose exposure. We are continuously exposed to N-nitroso compounds in air pollutants, for example, which are present in low concentration in the air we breathe. Mutagenicity testing strategies, in general, use a single large dose of test compound to induce the measurable effect, and hence do not account for chronic exposure. In an attempt to experimentally simulate chronic environmental exposure to DNA-damaging agents, Samson and Cairns, (1977) studied bacterial mutagenesis under conditions of constant low-dose alkylating agent exposure. The authors used this biological end-point since it has been previously demonstrated that there is a high correlation between mutagenesis and carcinogenesis i.e. the somatic mutation theory of carcinogenesis. The object of their study was to determine whether mutation induction occured with the frequency and kinetics one would expect from the more usual mutagenesis studies that involved the exposure of cell to extremely large doses of mutagen for a short period. They compared mutations induced during several days of mutagen exposure with those induced during several minutes of mutagen exposure. Since alkylating agents, particularly N-nitroso compounds, represent one of the

major classes of DNA-damaging agents in the environment these workers chose the simple monofunctional methylating agent MNNG as their study mutagen. The results were totally unexpected. An *E.coli* population, growing in the presence of a low, non-toxic MNNG dose (1 $\mu g ml^{-1}$), did not continuously accumulate mutant cells over a period of three days, since it had become resistant to mutation induction during the first 60 minutes of exposure. The mutation frequency did not increase any further during the remaining 4-7 days of the experiment. Further experimentation revealed that exposure of *E.coli* to sub-lethal levels of MNNG induced *de novo* synthesis of at least two DNA repair enzymes i.e. the response was absent when the experiment was performed in the presence of chloramphenicol, a protein synthesis inhibitor. These enzymes provided protection against the killing and mutagenic effects of further alkylation damage, even by very high challenge doses of MNNG (Samson and Cairns, 1977). This response was termed "The adaptive response to alkylating agents". Its characterisation by several laboratories, using a variety of biochemical, bacterial and molecular biology techniques, has allowed a fuller understanding of alkylation-induced cell death, mutation and the mechanisms bacteria can use to avoid these fates (Lindahl *et al.*, 1982, 1983).

This phenomenon raised a number of questions:

1) Does an analogous repair process exist in mammalian cells and if present, does it protect these cells from mutation and cytotoxicity induced by environmentally abundant alkylating agents, such as the N-nitroso compounds ?.
2) Could the existence of this response influence the approach taken toward the assessment of the mutagenic and carcinogenic

risk of environmental chemicals ?.

3) Since many of the drugs used in cancer chemotherapy are, or are metabolised to, alkylating agents, will the appearance of an adaptive response during these treatments, often pursued for long periods, modify the efficiency of the treatment or result in the appearance of iatrogenic diseases ?.
4) Does this response exist in man, and if so how does it help to deal with mutagenic insults presented by the environment ?.

The initial work using *in vitro* mammalian cell cultures, indicated the presence of an adaptive response to alkylating agents in two cell types i.e. Chinese hamster ovary (CHO) and GM637 SV40-transformed normal human fibroblasts (Samson and Schwartz, 1980). This group used alkylating agent-induced cytotoxicity and sister chromatid exchanges as their biological end-points. Studies using whole animal systems were also promising. Existance of a mammalian adaptive response was implicated when administration of a wide variety of N-nitroso compounds to rats, was shown to increase the O^6-methylguanine removal ability of liver extracts (1.4.3.).

A wealth of information about this response has subsequently been collected by many authors. They have used many different cell types, adaptation protocols and biological end-points. However, for an adequate description of the mammalian adaptive response, as it currently stands, it is beneficial to firstly present the main features of the better defined bacterial adaptive response.

1.2. The adaptive response in *E.coli.*

The work of Samson and Cairns, (1977) had shown that there were two main features of this response:
i) the repair system is inducible and ii) since it makes the cells less mutable, it is error-free. Schendel and Robins, (1978) ruled out the possibility of diminished accessibility of adapted cells' DNA to MNNG and it soon became evident that the resistance of induced cells could be correlated to a significant reduction of O^6-methylguanine (O^6MG) in their DNA. This repair process was found to be of limited use to the cell since the system became saturated at high doses of MNNG. This was explained by the discovery that the enzyme which mediated the reduction of O^6MG, is apparently expended in the reaction i.e. it is not catalytic in the usual sense of the term, and once the induced pool was exhausted the adapted cells became as unprotected as the unadapted ones (Robins and Cairns, 1979). After enzyme depletion both O^6MG and mutations started to accumulate in adapted cells (Schendel and Robins, 1978). Karran *et al*, (1979), showed that the disappearance of O^6MG from DNA was not accompanied by a corresponding release of the alkylated base. This is apparently due to the enzymatic transfer of methyl groups, from the methylated base, to a protein cysteine residue giving rise to S-methylcysteine in the enzyme structure (Olsson and Lindahl, 1980). The same group also suggested that the protein carrying the methyl acceptor and the methyltransferase activity resided in the same protein molecules since electrophoretic separation of the two proteins was not possible. The methyltransferase, therefore, is an enzyme that modifies itself in the course of the reaction it catalyses i.e. it

exhibits 'suicide' enzyme kinetics. Mutagenic adaptation involves the induction of this enzyme, called O^6-methylguanine-DNA-methyltransferase (O^6MGMT), with up to a 100-fold increase in the enzyme levels of adapted *E.coli* (Lindahl, 1982) and a corresponding 6000-fold decrease in mutation rate. (Cairns *et al.*, 1981). In each case mutation was assessed by reverse mutation of histidine dependent bacteria (*his-*) to histidine independent bacteria (*his+*).

O^6MGMT has been purified to 95% homogeneity (Demple *et al.*, 1983) from an *E.coli* mutant which constitutively expresses the adaptive response to alkylation-induced mutation i.e. *E.coli adc-1* (Sedgwick, 1982). Sodium-dodecylsulphate gel electrophoresis of the purified protein shows a single polypeptide with a molecular weight of approximately 18,000 Daltons (Olsson and Lindahl, 1980). The active form of this enzyme is also monomeric since non-denaturing conditions i.e. velocity sedimentation analysis, yields a similar molecular weight. Protease digestion followed by amino acid analysis indicates the presence of four (or possibly five) cysteine residues per molecule, but only one of these is the methyl acceptor (Demple *et al.*, 1983)

Under adaptive conditions, *E.coli* has demonstrated mutagenic resistance to ethylating, propylating and butylating agents, as well as against methylating agents. (Schendel, 1981). It should be noted, however, that adducts larger than methyl groups at the O^6 position of guanine in the DNA of *E.coli* can also be repaired by a process called excision repair - a topic not dealt with in this thesis.

The other inducible enzyme associated with this response, i.e. 3-methyladenine-DNA-glycosylase II (3-MAG), protects adapted

cells from mutagen-induced cell killing (Eveson and Seeberg, 1982; Karran *et al.*, 1982). Jeggo *et al.*, (1978) reasoned that mutagenic and lethal adaptation were two distinct pathways, when the DNA-polymerase-1 deficient *E.coli pol A* strains failed to acquire lethal resistance whilst exhibiting mutagenic resistance. The 3-MAG enzyme has been shown to be capable of removing 3-methyladenine, 3-methylguanine and 7-methylguanine from alkylated DNA (Riazuddin and Lindahl, 1978). The constitutively expressed 3-methyladenine-DNA-glycosylase I enzyme efficiently removes 3-methyladenine but not 7- or 3-methylguanine and since 7-methylguanine appears to be a relatively inocuous lesion, it was proposed that repair of 3-methylguanine is involved adaptation to mutagen-induced cell killing, (Karran *et al.*, 1982). These authors also showed that in *E.coli*, under adaptive conditions, glycosylase II is present in about 20-fold higher levels than non-adapted cells. A comprehensive explanation of general glycosylase-mediated repair is given in Friedberg, (1985).

The genetic control of the *E.coli* adaptive response has been studied in detail and is the subject of a number of reviews (Cairns *et al.*, 1981; Lindahl *et al.*, 1983; Sedgwick, 1982; Yarosh *et al.*, 1985) and will not be dealt with here.

It has been reported that *E.coli* contains O^2-methylthymine -DNA-glycosylase and O^4-methylthymine-DNA-methyltransferase enzymes (Ahmed and Laval, 1984), and that these identify with 3-MAG and O^6MGMT respectively (McCarthy *et al.*, 1984). These enzymes therefore appear to be able to remove several different kinds of lesion, with common structural features, from alkylated DNA.

cells from mutagen-induced cell killing (Eveson and Seeberg, 1982; Karran *et al.*, 1982). Jeggo *et al.*, (1978) reasoned that mutagenic and lethal adaptation were two distinct pathways, when the DNA-polymerase-1 deficient *E.coli pol A* strains failed to acquire lethal resistance whilst exhibiting mutagenic resistance. The 3-MAG enzyme has been shown to be capable of removing 3-methyladenine, 3-methylguanine and 7-methylguanine from alkylated DNA (Riazuddin and Lindahl, 1978). The constitutively expressed 3-methyladenine-DNA-glycosylase I enzyme efficiently removes 3-methyladenine but not 7- or 3-methylguanine and since 7-methylguanine appears to be a relatively inocuous lesion, it was proposed that repair of 3-methylguanine is involved adaptation to mutagen-induced cell killing, (Karran *et al.*, 1982). These authors also showed that in *E.coli*, under adaptive conditions, glycosylase II is present in about 20-fold higher levels than non-adapted cells. A comprehensive explanation of general glycosylase-mediated repair is given in Friedberg, (1985).

The genetic control of the *E.coli* adaptive response has been studied in detail and is the subject of a number of reviews (Cairns *et al.*, 1981; Lindahl *et al.*, 1983; Sedgwick, 1982; Yarosh *et al.*, 1985) and will not be dealt with here.

It has been reported that *E.coli* contains O^2-methylthymine -DNA-glycosylase and O^4-methylthymine-DNA-methyltransferase enzymes (Ahmed and Laval, 1984), and that these identify with 3-MAG and O^6MGMT respectively (McCarthy *et al.*, 1984). These enzymes therefore appear to be able to remove several different kinds of lesion with common structural features from alkylated DNA.

1.3. The adaptive response in other prokaryotic cells.

Bacillus subtilis and *Bacillus thurigiensis* have both exhibited the presence of an adaptive response to MNNG following pre-treatment with sub-toxic doses of this mutagen (Boutibones and Auffrey, 1987; Hadden *et al.*, 1983). Cell extracts of each strain contained methyltransferase activity that appeared to be very similar to that of the enzyme found in *E.coli*. In adapted *B.subtilis*, enzyme activity increases by an order of magnitude, again as a result of *de novo* protein synthesis following MNNG adaptive pre-treatment (Hadden *et al.*, 1983). In contrast, these authors failed to show an adaptive response in *Haemophilus influenzae*.

1.4. The adaptive response in mammalian cells.

1.4.1. The mammalian 3-methyladenine-DNA-glycosylase.

Evidence for the existence of a mammalian 3-methyladenine-DNA-glycosylase II enzyme, comparable to the bacterial enzyme, is sparse. However, Cathcart and Goldthwait, (1981) purified an enzyme from rat liver, that exhibited 3-MAG activity since it released 3-methyladenine and 7-methylguanine from alkylated DNA. Brent, (1979) purified an enzyme from human lymphoblasts that had 3-MAG activity and was specific for 3-methyladenine in duplex DNA.

It has been shown that a enzymic activity similar to that of 3-MAG is induced in rat liver during the regeneration period following partial hepatectomy (Gombar *et al.*, 1981). There is no direct evidence, however, that any of the above enzymes are analogous to the 3-methyladenine-DNA-glycosylase II of *E.coli*. The increased enzyme activity observed by Gombar *et al.*, (1981)

may simply reflect the increased proliferation of cells associated with liver regeneration.

1.4.2. The mammalian O^6-methylguanine-DNA-methyltransferase (O^6MGMT).

An enzyme activity similar to the O^6MGMT of *E.coli* has been found in a large variety of animal and human organs and tissues. This enzyme is also referred to as 'alkyl acceptor protein' and 'O^6-methylguanine-DNA-transmethylase'. Table 1.2. lists the various tissues reported to contain this enzyme (mostly *in vitro* cell culture systems) and also the corresponding literature reference.

Numerous methods for quantifying the activity of this enzyme exist but the indirect methods, rather than the direct assay methods, are the most frequently used. Monitoring the disappearance of O^6MG from an exogenous alkylated DNA substrate, following incubation with tissue extracts or other enzyme preparation, is a commonly used assay. Substrates used include ^{3}H-methylated calf thymus DNA (Brent, 1986; Frosina and Laval, 1987), *Micrococcus luteus* DNA (Karran *et al.*, 1979; Samson and Linn, 1987) or the synthetic polymer poly(dC.dG[^{3}H]O^6medG) (Boiteux and Laval, 1985; Foote *et al.*, 1983). O^6MG is measured by HPLC analysis, gel electrophoresis or by radioimmunoassay. Using the synthetic polymer, containing O^6MG labelled in the purine ring, the *in situ* conversion of O^6MG to guanine can be followed (Boiteux and Laval, 1985; Foote *et al.*, 1983; Pegg *et al.*, 1983).

Direct methods involve the measurement of protein-bound radioactivity associated with the methylated (inactivated) enzyme (Pegg *et al.*, 1983; Yarosh *et al.*, 1984). Protease digestion and subsequent amino acid separation allows the amount of

Table 1.2. Evidence for the presence of O^6MGMT in mammalian tissues.

Source tissue.	Reference.
Rat liver	Den Englese *et al.*, 1986; Margison *et al.*, 1984.
Rat hepatoma	Frosina and Laval, 1987; Lefebvre and Laval, 1986.
Rat kidney	Foote and Mitra, 1984.
Rat lung	Margison *et al.*, 1985.
Mouse liver	Bogden *et al.*, 1981.
Mouse prostate	Smith and Margison, 1981.
Mongolian gerbil	Bambourschke *et al.*, 1983.
Monkey liver	Hall *et al.*, 1985.
Human liver	Hall *et al.*, 1985; Pegg *et al.*, 1982.
Human lymphocytes	Setlow *et al.*, 1982; Waldstein *et al.*, 1982a.
Human skin fibroblasts	Karran *et al.*, 1982; Domoradzki *et al.*, 1985; Yarosh *et al.*, 1986.
Human brain	Gerson *et al.*, 1986.
Human foetal tissues	Krokan *et al.*, 1983.
Human placenta	Yarosh *et al.*, 1984.
HeLa cells	Dolan *et al.*, 1985; Foote and Mitra, 1984; Waldstein *et al.*, 1982c.
Chinese hamster lines	Foote and Mitra, 1984; Waldstein *et al.*, 1982b; Margison *et al.*, 1982.

S-methylcysteine to be measured (Craddock *et al.*, 1982)

The O^6MGMT enzyme has been partially purified from regenerating rat liver (Pegg *et al.*, 1983) and human placenta (Yarosh *et al.*, 1984). The structural and kinetic properties of this enzyme are similar to those described for the *E.coli* O^6MGMT enzyme described in 1.2. The protein is monomeric with an estimated molecular weight of between 20,000 and 22,000 Daltons (Harris *et al.*, 1983; Pegg *et al.*, 1983), which is close to the value of 18,000 quoted for *E.coli* (Olsson and Lindahl, 1980). There is a stoichiometric relationship between the amount of methyl groups removed from DNA and the amount of enzyme molecule used. In a similar fashion to the *E.coli* enzyme, a methyl group of O^6MG, in alkylated DNA, is transfered to a cysteine residue on the enzyme itself. This results in the generation of a guanine residue and S-methylcysteine followed by total protein inactivation (Bogden *et al.*, 1981; Craddock *et al.*, 1982; Pegg *et al.*, 1983; Waldstein, 1982a). The stoichiometric nature of the alkyl transfer reaction has allowed an approximate calculation of the number of alkyl acceptor sites per cell. Harris *et al.*, (1983) estimated 10,000 to 25,000 molecules per cell in Raji human lymphoma cells, whilst Foote *et al.*, (1983) found 100,000 in HeLa CCL2 cells. Waldstein, (1982c) demonstrated a 3-fold increase in enzyme levels (to over 300,000 molecules per cell) after adaptive pre-treatment of the same HeLa cell line. In fully adapted *E.coli* cells, with a genome of $3x10^6$ base pairs, there are approximately 3000 O^6MGMT molecules per cell i.e. 1 protein molecule per $1x10^3$ base pairs (Lindahl, 1982). HeLa CCL2 cells, under the same optimal adaptive conditions however, were shown to contain approximately 100 times as much enzyme , and with an approximate genome of $6x10^9$ base pairs, a

ratio of 1 protein molecule to $2x10^4$ base pairs is seen. There appears to be a 20-fold difference between bacteria and mammalian cells, under adaptive conditions, in the efficiency of this repair process. Under non-adaptive conditions, however, the repair potential of each is similar if the DNA content is again taken into account i.e. 1 protein molecule per $1.5x10^5$ base pairs, in *E.coli* and 1 protein molecule per $6x10^4$ base pairs, in HeLa cells.

Laval and Laval, (1984) reported an increase in the O^6MGMT activity of rat hepatoma H_4 cells, following a single exposure to MNNG, MMS or EMS. MNNG pre-treatment caused an initial decrease in the enzyme level but *de novo* protein synthesis reversed this, resulting in a 3-fold increase over the constitutive enzyme level after 48 hours. MMS and EMS did not cause this initial decrease leading the authors to conclude that pre-treatment caused the measured induction rather than enzyme disequilibrium caused by re-synthesis. Durrant *et al.*, (1981), using V79 cells reported no increase in the ability to demethylate O^6MG following a single non-toxic dose of MNU.

Studies utilising *in vitro* mammalian cell cultures have revealed two opposite phenotypic characteristics with regard to their ability to repair alkylation damage. The Mer^+ phenotype is assigned to cells which express methyltransferase activity (methyl excision repair proficient) and the Mer^- phenotype to transferase-deficient cells (Day *et al.*, 1980; Foote *et al.*, 1983; Goth-Goldstein and Hughes, 1987a; Yagi *et al.*, 1984). This phenomenon was first observed by Day *et al.*, (1980) who defined this phenotype as the competence of cells to support the growth of MNNG-treated adenovirus 5. Mer^- cells are deficient in the ability to reactivate this virus but are able to reactivate UV irradiated

viruses. Cells of this phenotype also show a greater sensitivity to the cytotoxic effects of methylating agents compared to Mer$^+$ cells (Day *et al.*, 1980). The Mer phenotype appears to correlate with a phenotype designated Mex, which distinguishes cells that effect removal of O^{6}MG from their DNA (Mex$^+$), from those that do not (Mex$^-$) (Sklar and Strauss, 1983). The distinction between Mer$^+$ and Mer$^-$ (or Mex$^+$ and Mex$^-$) cells is not always clear and intermediate degrees of removal ability have been reported (Day *et al.*, 1980; Yarosh *et al.*, 1983).

The observation that the ability to remove O^{6}MG always correlated with resistance to MNNG, seemed to indicate that O^{6}MG is a lethal lesion (Domoradzki *et al.*, 1984). The HeLa MR cell variant (Mer$^-$), which is hypersensitive to alkylation killing and mutagenesis, was found to repair O^{6}MG with much lower efficiency than the Mer$^+$ HeLa CCL2 cell line (Baker *et al.*, 1979; Foote *et al.*, 1983). A recent study, however, showed that HeLa Mer$^-$ cells that have survived a highly toxic dose of MNNG, regain Mer$^+$ resistance to the cytotoxic effects of MNNG but without simultaneously acquiring tha ability to repair O^{6}MG (Goth-Goldstein and Hughes, 1987a). Scudiero *et al.*, (1984) suggested that the two characteristics commonly associated with the Mer$^+$ phenotype i.e. O^{6}MG repair and resistance to MNNG, can be separated and the latter defined by the Rem phenotype. The Rem phenotye originated from the observation that some Mer$^+$ tumour cell strains show intermediate sensitivity to the cytotxic effects of MNNG, between that of Mer$^-$ and Mer$^+$ lines. They termed these strains Mer$^+$ Rem$^-$, defining the Rem phenotye by 'resistance to MNNG-produced cell killing'. An example of a cell line which corresponds to this phenotype is the human lung carcinoma line

A549, described by Yagi *et al.*, (1984), which was found to possess the ability to repair O^6MG lesions but was sensitive to MNNG-produced cell killing. In contrast, mouse mammary tumour line 34I lacks the ability to repair O^6MG but was relatively more resistant to MNNG than other Mer^- lines and is therefore an example of a Mer^- Rem^+ phenotype. A list of over 80 cell lines investigated for the Mer^+/Mer^- phenotype, is given in Frosina and Abbondandolo, (1985). The Mer designation is essentially erroneous since the O^6MGMT protein does not actually repair methylation damage via an excision process. A more correct designation of this phenotype could be MDR, representing methylation damage repair.

1.4.3. Animal studies.

Margison *et al.*, (1984) have reviewed the results of a great number of studies which have been carried out on rodents, assessing the inducibility of the O^6MG repair system by low doses of alkylating agents. The tabulated evidence was conflicting e.g. inducible enzyme acivity was found in rat liver but not in other rat tissues, and this activity was induced by a wide variety of N-nitroso compounds but not by directly acting alkylating agents such as MNU and MMS (Table 1.2.). The enhancement of O^6MG repair, as seen in rat liver, was not reproducible in other rodent models. The increase in O^6MG repair capacity, however, was not exclusive to alkylating agents since it was found that non-alkylating, hepatotoxic agents e.g. 2-acetylaminofluorine (AAF), ionising radiation and even partial hepatectomy or unilateral nephrectomy could also stimulate O^6MG repair (Cooper *et al.*, 1982; Margison *et al.*, 1985; Pegg and Weist, 1983).

Table 1.3. The inducibility of O^6MGMT in whole animal systems.

Tissue.	Inducing agent.	Increase in O^6MG repair capacity.	Reference.
Rat liver	DMN	+	Margison *et al.*, 1985.
	DMH	+	Bedell *et al.*, 1982.
	DEN	+	Margison *et al.*, 1980.
	DPN, DBN	+	Margison, 1982.
	AAF	+	Cooper *et al.*, 1982.
	AFB_1	+	Chu *et al.*, 1981.
	MNU	-	Margison *et al.*, 1980.
	MMS	-	O'Connor and Margison, 1981.
Rat lung	DMN	-	Margison *et al.*, 1985.
Rat kidney	DMN	-	Margison *et al.*, 1980.
Mouse	DMN	-	Lindamood *et al.*, 1984.
Chinese hamster	DMN	-	Smith and Margison, 1981.
Syrian hamster	DMN	-	Margison *et al.*, 1985.
Mongolian gerbil	DMN	-	Bambourschke *et al.*, 1983.

Abbreviations: DMN = dimethylnitrosamine; DMH = 1-2-dimethylhydrazine; DEN = diethylnitrosamine; DPN, DBN = dipropyl- and dibutylnitrosamine; AAF = 2-acetylaminofluorine; AFB_1 = aflatoxin B_1; MNU = N-methyl-N-nitrosourea; MMS = methylmethanesulphonate.

1.4.4. Mutation and related effects.

1.4.4.1. Mutation.

To live and to multiply, organisms depend on the information encoded in their genes (the units of inheritance). The material of inheritance of all living organisms, with the exception of some viruses, is carried by deoxyribonucleic acid (DNA). The normal DNA molecule is a linear macromolecule consisting of a pair of polynucleotide strands coiled around each other in the form of a double helix. The two strands are held together by hydrogen bonds between pairs of purine (adenine (A) and guanine (G)) and pyrimidine (cytosine (C) an thymine (T)) bases. Base pairing is subject to a number of physical and chemical constraints allowing A to bond only with T, and G only with C (Figure 1.1.). The AT and GC base pairs therefore enforce complementarity between opposite strands of the duplex. In order to make identical copies of itself for eventual distribution to daughter cells, the two DNA strands separate to allow pairing with incoming complementary mono-deoxyribonucleotides. Each strand serves as a template for the formation of a new daughter strand by normal AT and GC base pairing. The replicated region of DNA consists of two duplexes, each having a parental strand paired with a newly-synthesised daughter strand. The process of replication is therefore semi-conservative, since each strand of the parental duplex is conserved as a partner to each of the daughter strands.

The order of bases on each template strand constitutes a genetic message which contains all the information necessary to determine the specific structure and function of that cell. In a complex process involving messenger RNA, ribosomes and transfer

RNA, the genetic message is translated to yield a specific polypeptide chain (Watson, 1976). Each amino acid component of a particular polypeptide is specified by a triplet of successive bases (a codon) in the DNA. A linear sequence of codons, which code for a polypeptide, is called a gene.

A gene mutation is a heritable alteration in the sequence or number of bases in DNA. Mutation may occur spontaneously or may be induced by physical or chemical agents. Gene mutation can occur by base substitution i.e. one base is substituted by another, or by deletion or addition of one or more bases from one or more codons. Mutations due to substituions are known as base-pair substituions and those due to additions or deletions as frameshift mutations.

Base-pair substitution mutations occur when an incorrect base is inserted, e.g. by pairing with an alkylated natural base, which then pairs with its natural partner during DNA replication. In this way a new pair of incorrect bases are inserted into DNA. This results in the alteration of an amino acid within the polypeptide gene product. When a frameshift mutation occurs, however, the defect in the newly-synthesised polypeptide may be positioned at a site crucial to the integrity of the protein e.g. at a folding point or active site of an enzyme. Frameshift mutations are usually expressed as decreases in function rather than by total loss, and can often be identified by this property. Gene mutations affecting dispensible, enzymatic functions, can be detected if the resultant mutant cell has a phenotypic trait which allows it to be distinguished from normal (wild-type) cells.

Gene mutations are classified as being either 'forward' (wild type to mutant) or reverse (mutant to wild type). Reverse

mutation is generally studied in mutant cells containing known base-pair substitutions or frameshift mutations. Normal functional activity can be restored to these cells by a new substitution, a second addition or deletion close to the original change. A reverse mutation, therefore, requires a highly specific type of DNA interaction. The best known, i.e. the most widely used, test measuring reverse mutation, is the Ames test. Ames *et al.*, (1973), devised a simple test for the screening of environmental chemicals for mutagenicity, based on the reversion of histidine requiring *Salmonella typhimurium* bacteria to prototrophy (growth factor independence) i.e. *his-* to *his+*.

Forward mutations may arise from substituions, additions or deletions of the bases of a gene. These can be detected when there is a change in an enzyme function resulting in auxotrophy (growth factor dependence), or in resistance to various chemical or physical agents. Genetic markers, based on forward mutations to drug resistance, are employed in mammalian cell mutation assays. The most commonly used markers are resistance to the antimetabolite 6-thioguanine (TG) (Bradley *et al.*, 1981; Hsie *et al.*, 1981), which detects both frameshift and base-pair substitutions, and resistance to the steroid compound ouabain (Baker *et al.*, 1974; Cole and Arlett, 1976), which detects only base-pair substitutions. The biochemical basis of the resistance to the cytotoxic actions of the purine analogue 6-thioguanine (TG) is well established and, has been extensively reviewed by Caskey and Kruh, (1979). Briefly, TG is converted into the nucleoside 5'-monophosphate, thioguanosine monophosphate, by the enzyme hypoxanthine-guanine-phosphoribosyl-transferase (E.C.2.4.2.8), abbrieviated as HGPRT, present in the purine

salvage pathway. After further phosphorylation this can then be incorporated into nucleic acids in place of guanine. Although sufficiently similar to guanine, to be metabolised in an identical manner, TG is sufficiently dissimilar to functionally alter the DNA and RNA into which it is incorporated and thus causes cell death. Resistance to TG arises, therefore, by a deficiency or inactivity of the HGPRT enzyme, induced by a mutagen or another process, leading to the inability of the cells to metabolise this exogenous purine (Hsie *et al.*, 1975). The biochemical basis underlying cellular resistance to ouabain, is discussed later in 4.2.

Chromosomal mutations, usually referred to as chromosomal aberrations, are much less delicate than gene mutations and are recognised as morphological alterations in the gross structure of chromosomes. In eukaryotic cells, many of the aberrations are observable by microscopic examination of fixed and stained metaphase cells. Aberrations include deletions, duplications and inversions of sections within chromosomes, and exchanges (or translocations) of sections between chromosomes. The more subtle changes are easily seen if the chromosomes are stained in special ways e.g. G-banding. Mutagen-induced sister chromatid exchanges (SCE) can be visualised by the FPG technique (Perry and Wolff, 1974) described in Chapter 7.

Numerical chromosome aberrations i.e. genomic mutations, involve changes in the number of chromosomes in the genome (ploidy changes). Such changes can result in polyploid genomes, where the normal diploid (euploid) genome is doubled or trebled. Loss or gain of a single chromosome is known as aneuploidy and may occur as a result of non-disjunction during cell division. Addition or

deletion of a single chromosome results in conditions called trisomy and monosomy, respectively.

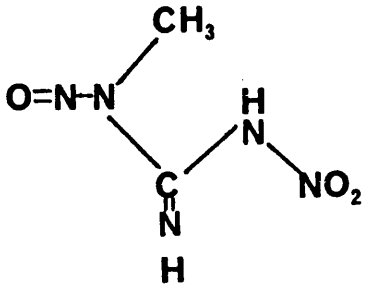
1.4.4.2. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

As previously mentioned, alkylating agents represent the largest class of mutagens present in the human environment (Fishbein *et al.*, 1979). Chemicals comprising a large proportion of this class are the N-nitroso compounds. Therefore to investigate the effect of long-term exposure of cells to N-nitroso compounds, and consequently the possible induction of the adaptive response, the simple monofunctional alkylating agent MNNG was chosen as a representative compound of this group. MNNG was the mutagen used by Samson and Cairns, (1977) in their initial studies into the adaptive response of *E.coli*. It has been used subsequently in *in vitro* studies into the mammalian adaptive response (Frosina and Laval, 1987; Lefebvre and Laval, 1986; Samson and Schwartz, 1980). One other reason for the choice of this mutagen was the fact that it does not need an energy-requiring cellular metabolic process to produce a reactive metabolite (metabolic activation) (Singer, 1979). This characteristic was attractive since it simplified the experimental aspects of mutagen dose administration, particularly during adaptive pre-treatment (Chapters 3 to 7).

MNNG is capable of inducing gene mutations in bacteria, yeast and mammalian cells (Auerbach, 1976; Kao and Puck, 1968; O'Neill *et al.*, 1977). It also induces structural and numerical chromosome aberrations in mammalian cells (Kao and Puck, 1969) and is a potent carcinogen (McCann *et al.*, 1975). MNNG is a monofunctional alkylating agent which is able to donate a single methyl group to nucleophilic sites within a biological

macromolecule. The structure of MNNG is shown below.

MNNG

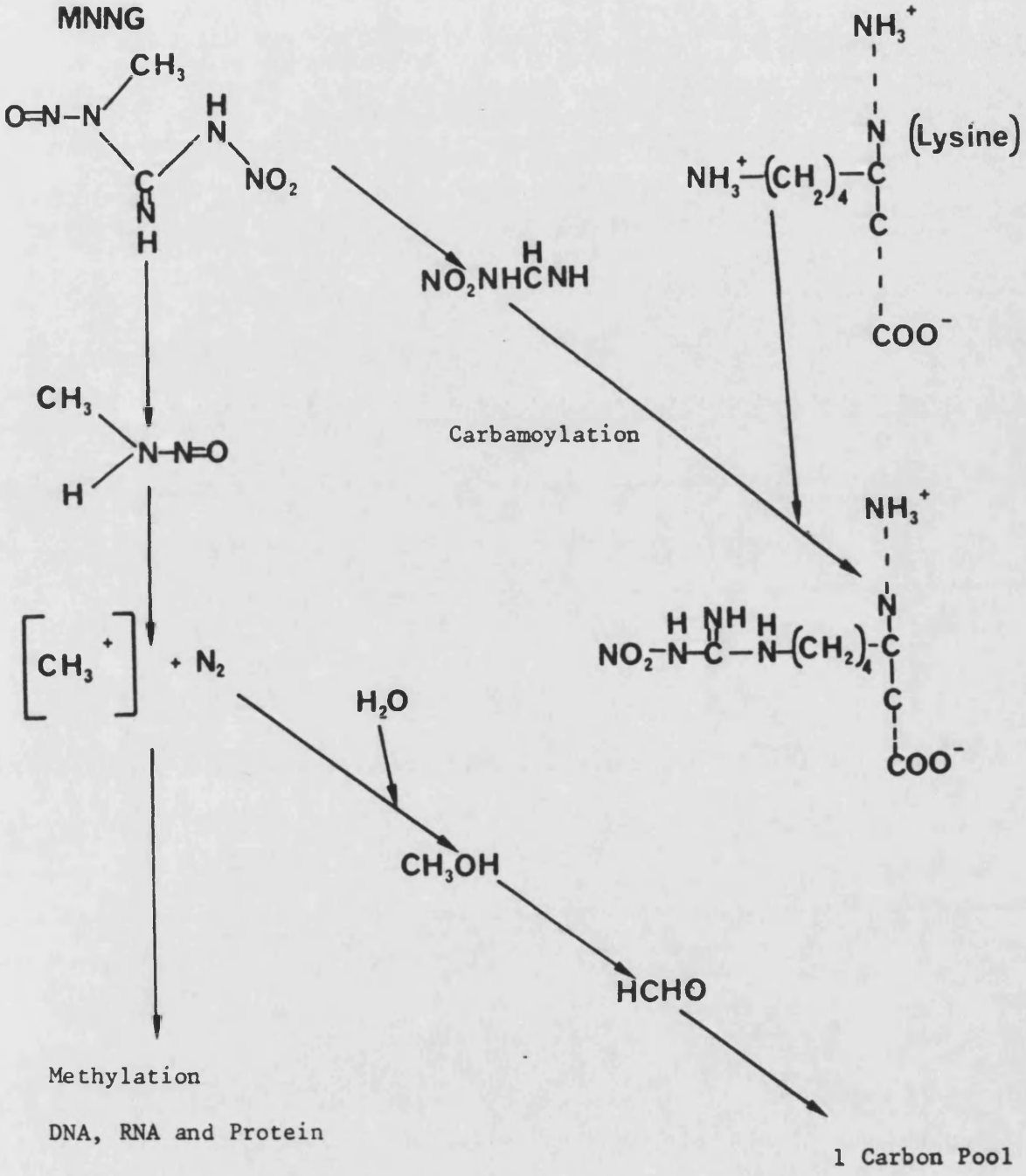


M.Wt. = 147.1

Most N-nitroso compounds, including MNNG, exert their biological effects via a highly reactive carbonium ion i.e. an ion in which one carbon atom has three bonds and possesses a positive charge. MNNG requires a thiol or primary amine to displace its carbonium ion i.e. the methyl diazonium ion (Lawley and Thatcher, 1970). This ion preferentially reacts with weakly nucleophilic centres in DNA and RNA, such as oxygen atoms, over strongly nucleophilic centres such as nitrogen atoms (Grisham and Smith, 1984). Figure 1.2. shows the position of DNA and RNA atoms susceptible to methylation by MNNG and also a suggested schematic respresentation of some possible chemical reactions of this compound with cellular constituents (Grisham and Smith, 1984). Besides methylating DNA and RNA, MNNG can cause carbamoylation of cellular proteins via the N-nitroguanido moiety (McKay, 1948) and has been shown to inactivate DNA polymerase by carbamoylation (Drahovsky and Wacker, 1975). Guanidation has been suggested as the cause of methylation induced malfunction of other enzymes (Kann *et al.*, 1974), including DNA repair enzymes.

Alkylation of DNA can produce potentially miscoding

Figure 1.2. Schematic representation of some possible chemical reactions of MNNG with cellular constituents.

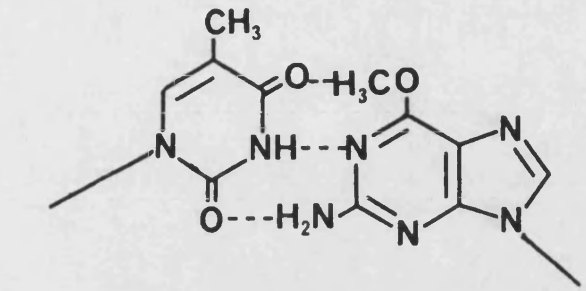


(mutagenic) lesions and potentially lethal lesions. O^6alkylG and O^4-alkylT probably represent the most important DNA lesions with respect to the induction of gene mutations, and cancer, by simple alkylating agents such as MNNG (Fox and Carlton, 1984; Lawley, 1974; Singer and Kusmierek, 1982). These alkylated bases almost certainly undergo mispairing with thymine and guanine, respectively, to generate GC to AT and AT to GC base-pair transitions (Drake and Baltz, 1976; Murray, 1987). Alkylation at the N^7 and N^3 position of guanine and the N^3 of adenine have been correlated with the lethality of MNNG (Karran *et al.*, 1982; Lawley, 1974). Some potential MNNG-induced alkylations, which are likely to lead to mispairing events, are shown in Figure 1.3. MNNG may also induce mutation indirectly by a misrepair mechanism, which occurs when a pre-mutational lesion interrupts daughter strand elongation during DNA replication (Drake and Baltz, 1976). The gap produced in the daughter strand becomes a substrate for an enzymic, error-prone post-replication DNA repair mechanism (Heidelberger, 1975; Hart *et al.*, 1978). When these gaps are filled either base-pair substitutions or frameshift mutations can be generated.

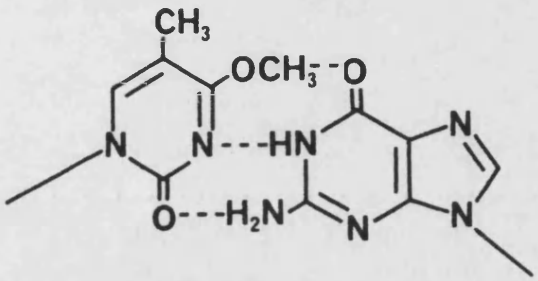
1.4.3. Mutagenic adaptation.

The results of experimental work to establish whether mammalian cells in culture possess a mutagenic adaptive response, analogous to that found in *E.coli*, have so far been conflicting. The interpretation of results obtained from a range of mutagenic end-points and the use of different adaptive pre-treatment protocols, is further complicated by the use of a wide variety of cell types. Mutagenic end-points so far used to assess the effect

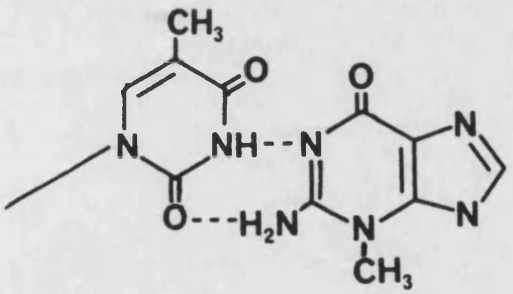
Figure 1.3. Some possible anomalous base-pairings of alkylated bases.



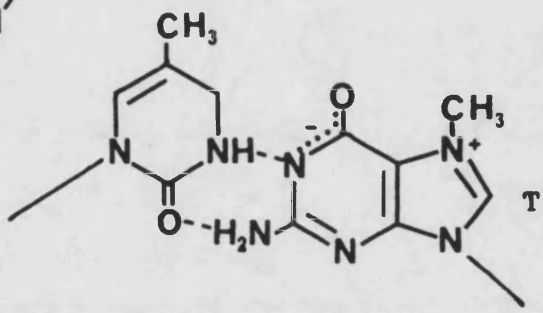
T : O^6MG



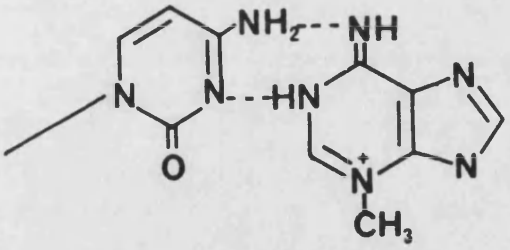
O^4MT : G



T : 3MG



T : 7MG



C : 3MA

Abbreviations: O^6MG = O^6-methylguanine; O^4MT = O^4-methylthymine; 3MG = N^3-methylguanine; 7MG = N^7-methylguanine; 3MA = N^3-methyladenine; G = guanine; C = cytosine; T = thymine.

of mutagen adaptive pre-treatment on cells are SCE induction, forward mutation to 6-thioguanine resistance ($HGPRT^+$ to $HGPRT^-$) or ouabain resistance (Oua^R), reverse mutation to HAT resistance (hypoxanthine, aminopterin and thymidine resistance , $HGPRT^-$ to $HGPRT^+$), induced chromosome aberrations and micronuclei induction.

The first indication that mammalian cells possess a mutagenic adaptive pathway was shown by Samson and Schwartz, (1980) who reported that MNNG pre-treatment caused a decrease in SCE and cell killing in CHO cells, on subsequent challenge with MNNG or MNU. SCE were also decreased in the SV40-transformed human cell line, GM637. Further to this work many authors reported results which suggest the presence or absence of a mutagenic adaptive resonse in cultured cells. Evidence contradicting the SCE findings of Samson and Schwartz has not yet been put forward for CHO cells, but a single MNU pre-treatment of V79 cells did not alter the MNU-induced SCE (Fox *et al.*, 1982b). Kaina, (1982), using V79-C10 cells, reported that a substantial decrease in the frequency of MNNG or MNU induced 6-thioguanine resistant mutants was caused by pre-treatments with single low doses of the same agents. Reduction in MNNG-induced killing was reported in the H_4 rat hepatoma line following pre-treatment with multiple, non-toxic doses of MNNG (Laval, 1986; Laval and Laval, 1984). The same pre-treatment protocol caused a marked increase in the ability of these cells to remove O^6MG from their DNA. Schwartz and Samson, (1983), using the pre-treatment schedule that had previously lowered the induced SCE frequency, showed a lack of mutagenic adaptation in CHO cells. Further negative evidence was produced by Durrant *et al.*, (1981), who demonstrated no change in the MNU induced mutation to 6-thioguanine resistance in V79 cells

following a single pre-treatment dose of MNU. Both observations were later confirmed by Frosina *et al.*, (1984) who subjected cells to 49 different pre-treatment protocols and a variety of pre-treatment and challenge mutagens. Fox *et al.*, (1982a) reported no change in the MNU-induced mutation to ouabain resistance of V79 cells pre-treated with a single dose of the same mutagen. The same group later reported a decrease in MNNG or MMS-induced mutation to HAT^R of HGPRT$^-$ V79 cells, pre-treated with a single dose of either MNNG or MMS (Fox and Carlton, 1984). Since V79 cells have been shown to lack the O^6MGMT enzyme (Foote *et al.*, 1983; Foote and Mitra, 1984), the authors did not interpret their results in terms of the induction of this enzyme, but instead suggested that if O^4-methylthymine was responsible for the mutational event, then adaptive re-synthesis of an enzyme specific for this lesion could be induced so explaining the reduction in mutation frequency.

Reduction in the induction of chromosome aberrations and cytotoxicity were found by Kaina, (1982) who, in a later publication, showed induced aberrations were reduced to a maximum of 50% less than control values following MNNG or MNU pre-treatments (Kaina, 1983a).

Karran *et al.*, (1982) were unable to show adaptation to MNU-induced micronuclei formation and cytotoxicity in GM730 normal human fibroblasts after MNNG pre-treatment.

1.5. Aims of the present study.

Following the discovery and characterisation of the bacterial adaptive response, a number of questions were raised concerning the existence of a similar response in mammalian cells (1.1.). The data currently available for *in vitro* cultures are not

sufficient to answer these questions. Far from answering them, two more pertinent questions have been raised during the course of these studies; i) are the discrepancies reported due to different experimental protocols used, and 2) are the contrasting results due to the different cell types used ?.

A number of workers have stressed the importance of the inductive schedule (Durrant *et al.*, 1981; Karran *et al.*, 1982; Laval and Laval, 1984). However, even when the same schedule had been used, contrasting results were still obtained (Frosina *et al.*, 1984; Kaina, 1982). Laval and Laval, (1984) suggested that pre-treatment for a long period (8 doses over a 48 hour period) was necessary for the induction of an adaptive response but the results of Kaina, (1982, 1983a, b), who induced adaptation following a single mutagen dose, suggested otherwise.

The diverse origins of cell lines like Chinese hamster CHO and V79 cells, rat H_4 hepatoma cells and human tumour lines may be explanation enough for the contrasting results. However, when the same cell line was used in three different laboratories (Fox *et al.*, 1982a; Frosina *et al.*, 1984; Kaina, 1982, 1983a, b) conflicting results were again obtained. Baker *et al.*, 1979 reported that HeLa cell clones differ in sensitivity to monofunctional alkylating agents and offered a possible explanation for the inter-cell line discrepancies i.e. some sub-clones of some cell lines have lost the ability to remove O^6MG from their DNA

The overall aim of this project, therefore, was to compare the adaptive response in V79 and CHO cells subjected to adaptive protocols which had previously been shown to induce this response in each line. In this way differences in cell line and adaptive

pre-treatment protocol could be ruled out as a reason for the lack of an observed response. Further to the suggestion of Baker *et al.*, (1979), cell clones that are either sensitive or resistant to the cytotoxic action of MNNG would be isolated, for both cell lines, and the effect of adaptive pre-treatment on these cell lines assessed. The techniques employed throughout this project would be those previously used to define the mammalian response and included MNNG-induced cytotoxicity, mutation to ouabain resistance, SCE and the O^6MG de-methylation ability of cell-free extracts. Correlation of these factors, therefore, should assist the fuller understanding of the adaptive response in these mammalian cells.

The widespread use of both CHO and V79 cell lines in the mutagenicity testing of industrial, pharmaceutical and manufacturing compounds (Venitt and Parry, 1984), also influenced the choice of cell lines for the present studies.

Note in proof.

Since this introduction was written a significant review concerning the nature of the *E.coli* O^6MGMT enzyme has been reported by Lindahl, (1987). The protein was found to contain two functional activities which could be physically separated after treatment with low concentrations of trypsin. The C-terminal half of the protein serves to repair O^6MG residues in DNA and accounts for the anti-mutagenic effect of the adaptive response. The N-terminal half of the protein can also abstract an alkyl group from modified DNA but does so from phosphotriester lesions. The intact enzyme has a molecular weight of 39,000 Daltons with the C-terminal half having a weight of 19,000 Daltons.

Lindahl, T. (1987). Br. J. Cancer. 56. 91-95.

CHAPTER 2. MATERIALS AND METHODS.

2.1. Equipment.

Laminar flow hood. During the course of this work two different flow hoods were used for all aseptic manipulations.

(i) 2m, horizontal displacement type (Fell Clean Air (1971) Ltd., Newhaven, Sussex.).

(ii) 1.3m, vertical displacement type, Class II biological safety cabinet, Microflow model no. 20229 (MDH Ltd, Andover, Hampshire).

The surfaces of hoods were washed with 70% ethanol before and after use.

Incubator.

LEEC PF2 anhydric incubator with forced air circulation (Laboratory and Electrical Engineering Company, Nottingham). The thermostatic controls were adjusted to maintain a temperature of 37.5±1°C.

Centrifuges.

(i) Beckman bench centrifuge model No. TJ-6 (Beckman Industrial Estate Mervue, Galway, Ireland.).

(ii) MSE Micro Centaur (MSE Scientific Instruments, Crawley, Sussex.).

Adjustable replicating pipettes.

Pipette	Effective Volume range
Gilson Pipetman P20	2-20 µl
Gilson Pipetman P200	20-200 µl
Gilson Pipetman P1000	200-1000 µl
Gilson Pipetman P5000	1000-5000 µl

Pipettes with disposable polypropylene tips were obtained from

Anachem Ltd., Luton, Beds. Pipettes were periodically checked to ensure accurate and reproducible sample delivery. A gravimetric method was used in which 10 replicates of a set volume of water at 20°C were each weighed on an analytical balance.

Liquid nitrogen freezers.

Two types of storage unit were used to house the 2 ml polypropylene ampoules containing the stock cultures.

(i) Union Carbide model No. LR-33-10 (Union Carbide UK, Ltd., Cleveland.), in which the ampoules are held in the liquid N_2 refrigerant or in its overlying vapour.

(ii) Union Carbide model No. LR-40, in which the ampoules are shelved in the vapour phase of the liquid N_2.

Freezing unit.

Union Carbide BF-6 biological freezer, a plug-type device, designed for use with the LR-33-10 freezer. This unit is capable of cooling up to eight 2 ml ampoules, to below -70°C, at a cooling rate of between 0.5°C and 7°C min^{-1}.

Haemocytometer.

Standard double grid improved Neubauer-type blood cell haemocytometer, with coverslips (Fisons Ltd., Loughborough, Leics.).

Microscopes.

(i) For examination of growing cell cultures under phase contrast an inverted biological microscope Wild M40 (Wild Heerberg Ltd.,Heerberg, Switzerland) was used. This

instrument fitted with appropriate condensers and x10 and x20 objectives gives magnifications of x187 and x375 respectively.

(ii) For haemocytometer and other cytological preparations an Amplival microscope with mf basic body and 35mm camera attachment (Carl Zeiss Jena, CZ Scientific Instruments Ltd, Borham Wood, Herts), was used. With fitted condenser and both plain and phase-ringed plane field objectives (x16, x40 and x100), this instrument allows transmitted light and phase-contrast microscopy at magnifications of x256, x640 and x1600.

(iii) Binocular dissecting microscope. Standard microscope model (C.Baker Ltd., London), which allows magnification at x20.

Disposable Cell Culture Plasticware.

Tissue culture (T/C) grade disposable plasticware was obtained pre-sterilised from Sterilin Ltd., Feltham, England. Items used: 25cm^2 and 75cm^2 T/C flasks, 100mm x 16mm T/C tubes with screw caps, 50mm x 13mm and 90mm x 16mm T/C petri dishes with triple vents.

2 ml polypropylene screw-capped ampoules, for liquid N_2 cell storage, and 30ml universal containers with caps were obtained pre-sterilized from Sterilin Ltd.

Glassware.

100ml and 500ml bottles for the storage of solutions and media were obtained with caps from Flow Laboratories Ltd., Rickmansworth, Herts.

150ml and 500ml soda glass 'medical' flat bottles and caps,

and general laboratory glassware were obtained from Fisons Ltd.

2.2. Cell Lines.

(i) CHO-K1: a L-proline requiring Chinese hamster cell line (Kao and Puck, 1968) were isolated and characterised by R.S.Dewdney (1982). Genetic heterogeneity is markedly reduced in cell populations derived from single, cloned cells (Ham and Puck, 1962). These cells were used in experiments between passages 30 and 40.

(ii) CHO-K1S: an MNNG-sensitive sub-clone of CHO-K1, isolated as described in 5.3.2.

(iii) CHO-K1R: an MNNG-resistant sub-clone of CHO-K1, isolated as described in 5.3.1.

(iv) V79-379A: a sub-clone of V79-1, male Chinese Hamster lung fibroblasts (Ford and Yerganian, 1958), were obtained from Flow Labs Ltd. These were obtained at an unknown passage number and so were arbitrarily designated at passage 2, and were used between passage 2 and 12 in experiments.

(v) V79-379A/S: an MNNG sensitive sub-clone of V79-379A, isolated as described in 5.3.2.

(vi) V79-379A/R: an MNNG resistant sub-clone of V79-379A isolated as described in 5.3.1.

(vii) C3H $10T^1/_2$: mouse embryo fibroblasts originated in Charles Heidelbergers laboratories in Los Angeles (Reznikoff *et al.*, 1973), were kindly provided by Dr.T.Meyer (Shell Research, Sittingbourne, Kent). Cells were received at passage 10 and used in experiments between passages 10 and 20.

Paul, (1975) outlined the problem of cellular cross-contamination whilst maintaining more than one cell line within a

laboratory. Great care was therefore taken to maintain the purity of the cultures throughout these studies by avoiding simultaneous handling of different cell lines, and by sequestration of media for use with individual cell lines.

2.3 Cell Culture Materials.

2.3.1. Water.

Double glass distilled water (DDH_2O) was used in the preparation of all solutions and media. This was produced by a 6kW, 4.5 litre hr^{-1} bi-distillation Fi-stream still, model 2903 (Fisons Ltd.) fitted with a Fi-stream pre-deioniser (Fisons Ltd). DDH_2O was sterilised by autoclaving at 121°C for 15 minutes in 100ml and 500ml volumes. Sterile distilled water produced in this way has a pH of 4.5.

2.3.2. Balanced salt solution.

Dulbecco's phosphate buffered saline was routinely used, either with or without added calcium or magnesium ions. Solutions were designated either PBS or PBS(A) respectively.

Composition: (Dulbecco and Vogt, 1954)

Component	gl^{-1} DDH_2O		
NaCl	8		
KCl	0.2	PBS(A)	PBS
Na_2HPO_4	1.15	pH=7.3	pH=7.4
KH_2PO_4	0.2		
$CaCl_2.2H_2O$	0.1		
$MgCl_2.6H_2O$	0.1		

Sterile PBS(A) was prepared by dissolving one Dulbecco A

tablet (Oxoid Ltd,London) in 100ml of freshly prepared DDH_2O and autoclaving for 15 minutes at 121°C. PBS was prepared by the aseptic addition of 0.5ml Dulbecco B solution (Oxoid Ltd.) to 100 ml of sterile PBS(A). Both PBS and PBS(A) were stored at room temperature, for a maximum of one month.

2.3.3. Trypsin solution.

Trypsin (tryptic activity 1:250*) was obtained as a 2.5% w/v solution in Hanks balanced salt solution without calcium, magnesium or phenol red in pre-sterilised 100ml unit quantities from Flow Labs Ltd. This was aseptically diluted to working strengths of 0.025% w/v plus 0.02% w/v EDTA (Sigma) or 0.1% w/v plus 0.02% w/v EDTA, with PBS(A).(Working strengths quoted relate to CHO/V79 cell lines and the C3H $10T^1/_2$ cell line respectively). All solutions were stored at -20°C in approximately 16ml volumes in plastic universals and used within one month. Prior to use the solutions were thawed in a 37°C water bath.

(* 1:250 : one part of trypsin will convert 250 parts of casein to peptides, peptones and amino acids under the conditions of the US National Formulary assay for pancreatin (Vol 14, 1975)).

2.3.4. Additives for Cell Culture Media.

(i) Antibiotic solution: Penicillin (5000 IU ml^{-1}) and streptomycin (5000 $\mu g ml^{-1}$) was obtained as a sterile solution from Flow Labs Ltd in 100ml unit quantities. This was sub-divided into 15ml volumes and stored in plastic universals at -20°C for a maximum of one year.

(ii) L-glutamine: was obtained as a sterile 200mM solution in

100ml quantities from Flow Labs. Ltd. This was sub-divided into 15ml volumes and stored in plastic universals at -20°C for a maximum of one year.

(iii) L-proline: a 100mM solution was prepared from chromatographically homogenous L-proline (cell culture tested, Sigma chemicals Ltd., Poole, Dorset) in DDH_2O, and sterilised by membrane filtration. 15ml volumes of this solution were stored in plastic universals at -20°C for a maximum of one year.

(iv) Ethylene diamine tetra acetic acid (EDTA, versene): a 2% w/v solution of EDTA (Sigma Chemicals) was prepared in DDH_2O and sterilised by membrane filtration. 15ml volumes of this solution were stored in plastic universals at -20°C for a maximum of six months.

(v) Sodium bicarbonate: was obtained as a sterile 7.5% w/v $NaHCO_3$ solution in 100ml quantities from Flow Labs Ltd. This solution was stored at room temperature for a maximum of one year.

(vi) Hypoxanthine (6-hydroxypurine): 81.6mg of hypoxanthine, (minimum assay 99%, BDH Chemicals), were dissolved in 50ml of boiling DDH_2O, made alkaline with 2 drops of concentrated ammonium hydroxide solution and made up to a final volume of 1 litre with DDH_2O. The final solution (0.6mM) was filter sterilised, divided into 100ml volumes and stored at 4°C protected from light.

All solutions stored at -20°C were thawed in a water bath at 37°C immediately prior to use.

2.3.5. Media.

(i) Hams F10 medium: was obtained from Flow Labs Ltd. as a sterile 10x liquid concentrate containing phenol red but lacking L-glutamine and $NaHCO_3$. The concentrate was received in 500ml units and split into 100ml aliquots and stored at 4°C for a maximum of one year. 500ml volumes of single strength media were aseptically prepared from the 10x concentrate plus the media additives added in the order given below:

Component	Volume
DDH_2O	428.5ml
Hams F10	50ml
(10x concentrate)	
Antibiotic solution	5ml
L-proline (100mM)	1ml
L-glutamine (200mM)	7.5ml
$NaHCO_3$ (7.5% w/v)	8ml

Once prepared the medium was stored in 100ml volumes at 4°C and used within four weeks.

(ii) Hams F10 medium lacking hypoxanthine, thymidine and $NaHCO_3$: was obtained in powder form from Flow Labs Ltd. and stored at 4°C in a vacuum desicator. Powder to prepare one litre of single strength medium was added to approximately 900ml of freshly colected DDH_2O at room temperature. The powder was allowed to dissolve and the following components added in the order given:

Component	Volume
Antibiotic solution	10ml
L-proline (100mM)	2ml
L-glutamine (200mM)	10ml
Hypoxanthine (0.6mM)	50ml
$NaHCO_3$ (7.5% w/v)	16ml

The medium was made up to 1 litre with DDH_2O, rendered slightly acidic by bubbling CO_2 through it, then sterilised by positive pressure membrane filtration (2.5.1.). The sterilised medium was stored in 100ml volumes at 4°C and used within four weeks.

Note: Storage conditions and maximum storage times for cell culture materials above were those recommended in the Flow Laboratories catalogue.

2.3.6. Sera.

For the Chinese Hamster cell lines studied the culture medium was supplemented with 5% foetal calf serum (FCS), whereas the mouse C3H $10T^1/_2$ line was grown in medium supplemented with 10% FCS. To achieve levels of 5% or 10% either 5.3ml or 11.1 ml, of FCS, respectively, added to 100ml of medium immediately prior to use.

During the course of these studies FCS from two different batches was used. Both serum lots were obtained from Sera Labs Ltd., Crawley, Sussex, and selected for the ability, as a media supplement, to support clonal growth. Assessment of serum suitability, involved dilution plating of small numbers of CHO-K1 cells (2.6.7.) into T/C petri-dishes containing media supplemented

with different batches of test serum. Following 7 days incubation the colonies formed were stained, scored and a batch comparison made on the basis of relative colony size and plating efficiencies. Using these criteria, two batches were chosen from a total of six test batches supplied by Sera Labs Ltd.

Batch 1 : Sera Labs batch number 301110

Batch 2 : Sera Labs batch number 404010

Both serum batches were obtained filter sterilised, frozen in 500ml bottles which were allowed to thaw at 4°C, then sub-divided into 100ml volumes. These were stored frozen at -20°C for a maximum of 18 months. As required, 100ml volumes were withdrawn from stock and allowed to thaw at 4°C. Once thawed the serum was stored at 4°C and used within four weeks.

2.3.7. Cryoprotectants and Solvents.

(i) Glycerol, AnalaR grade (Fisons Ltd) was stored at room temperature, protected from moisture. Prior to use as a cryoprotectant, 2-3ml volumes were sealed in glass powder ampoules and sterilised in a hot air oven 160°C for one hour.

(ii) Dimethyl sulphoxide (DMSO), grade 1 (Sigma) was obtained in 100ml quantities and stored in tightly capped bottles at room temperature. DMSO was filter sterilised through 0.2 μm Millex-FG filter units.

(iii) Acetone, hplc grade (Fisons Ltd) was stored at room temperature, protected from moisture.

2.3.8. Biological Stains.

(i) Methylene blue (BDH Chemicals) was prepared as a 0.5% w/v solution in 50% v/v methanol. It was used to stain mammalian cell

colonies attached to T/C plates.

(ii) Giemsa stain, Improved R66 solution (Hopkin and Williams, Chadwell Heath, Essex.), was used as a 5% w/v solution in Sorensens buffer pH=6.8, for the differential staining of sister chromatids.

2.4. Mutagen and Mutation Selecting Agent.

2.4.1. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

This was obtained in crystalline form (Sigma) and stored in a desiccator at -20°C. Stock solutions of 1 $mgml^{-1}$ (6.8mM) (solubility limit 4 $mgml^{-1}$ (27mM)), were prepared by dissolving the solid in a small volume of acetone, not exceeding 0.5% v/v of the final volume, then making up to the required amount with DDH_2O. 10ml aliquots were sterilised by passage through 0.2 μm Millex-GS filter units. This solution was stored at -20°C, in darkness. Immediately before use stock solutions were thawed in a 37°C water bath and dilutied with sterile DDH_2O.

2.4.2. Ouabain. (G-strophanthin).

This was obtained as ouabain octahydrate crystals (Sigma) and stored at room temperature. Stock solutions were made at 10mM in culture medium by dissolving at 50°C, protected from light. Solutions were sterilised by membrane filtration, stored in the incubator at 37°C and used within 48 hours. 1ml of the stock solution was added to each selection plate with 9ml medium to give a final concentration of 1mM.

2.5. General Methods.

2.5.1. Sterilisation by Membrane Filtration.

Heat-labile liquids, solutions and media were sterilised by membrane filtration.

(i) Small volumes were filtered through autoclave-sterilsed 25mm Swinnex units (Millipore UK Ltd., London), fitted with 0.2 µm pore size Sartorius membrane filter discs, (V.A.Howe and Co. Ltd., London.).

(ii) Small volumes of hazardous solutions were filtered from luerlocked syringes through disposable Millex-GS units (Millipore), fitted with membrane filters of 0.2 µm pore size.

(iii) Non-aqueous solvents, e.g. DMSO, incompatible with cellulose nitrate filters, were filtered through disposable Millex-FG units (Millipore), fitted with Fluoropore hydrophobic membrane filters made of polytetrafluoroethylene (PTFE).

(iv) Large volumes of solutions and media i.e. greater than 250ml, were filtered from a positive pressure apparatus SM16616/80 (Sartorius GmbH, Gottingen, FRG.), through an autoclave-sterilised 47mm Swinnex unit fitted with an AP25 pre-filter (Millipore) and a 90mm, 0.2 µm pore size membrane filter enclosed in a sterile Sartorius filtration tripod unit type SM16260. Filtration under positive pressure rather than negative pressure was preferentially used as it prevents frothing and reduces the loss of CO_2 from bicarbonate containing media.

2.5.2. Recycling of Glassware.

Immediately after use all glassware was rinsed with tap water,

and, except for pipettes, processed as follows.

(i) Soaked in a hand-hot 2% v/v solution of RBS25 detergent (Fisons Ltd.) for 30 minutes.

(ii) Thoroughly cleaned, using China brush on all surfaces,in running water.

(iii) Rinsed in three changes of tap water, being left for 30 minutes in the last rinse.

(iv) Rinsed in three changes of distilled water, again leaving for 30 minutes in the last rinse.

(v) Finally, all articles were rinsed and left for at least 30 minutes in a large volume of freshly collected glass distilled water.

(vi) After drying in a hot air oven at 160°C, all items were foil-capped and sterilised in a hot air oven at 160°C for a minimum of one hour. Graduated glass pipettes were rinsed in a pipette washer with tap water, for at least 30 minutes, and then rinsed as above, from (iii). After drying in a hot air oven, pipettes were put into metal canisters and sterilised at 160°C for a minimum of one hour.

2.5.3. Recycling of Tips, Bottle Caps and other Non-glass items.

Immediately after use these were rinsed with tap water and cleaned by boiling in three changes of single distilled water. After this they were rinsed as for glassware (from 2.5.2 (iii)). After drying in a warm oven at 55°C, all items were sealed in autoclave bags (DRG Hospital Supplies, Bristol) and sterilised by autoclaving at 121°C for 15 minutes.

2.5.4. Siliconising of Pasteur Pipettes.

Pasteur pipettes were siliconised by a double rinse with a 2% w/v solution of dimethylchlorosilane in 1,1,1-trichloroethane (BDH Chemicals, Poole, Dorset.). After drying in a hot air oven at 160°C they were immersed in a large volume of freshly collected distilled water for a minimum of 30 minutes to remove any trace of hydrochloric acid formed in the treatment. After drying the now siliconised pipettes were put into metal canisters and sterilised in a hot air oven at 160°C for a minimum of one hour.

2.5.5. Gases and Gassing procedures.

(i) Cylinders of N_2, CO_2 and 5% CO_2 in air mixture (5% CO_2 and 20% O_2 in N_2) were all obtained from British Oxygen Company, Bristol. CO_2 and 5% CO_2 in air mixture were piped to each work station from a central holding reservoir.

(ii) Gas flowmeter, floating needle type, 0.1-1.0 $lmin^{-1}$ (Rotameter Manufacturing Co. Ltd., Croydon) calibrated for use with CO_2.

(iii) Incubation boxes, rigid clear plastic boxes, 3.25 litre volumes (A. Gallenkamp and Co. Ltd., London.).

(iv) Gas-tight tape, British standard vinyl tape, 2.5cm wide (Intech Tapes Ltd., Manchester).

The cell culture media used contain a bicarbonate pH buffer system. When sodium bicarbonate dissociates CO_2 is released into the atmosphere with a resultant increase in alkalinity. It is possible to control this reaction by artificially supplying carbon dioxide to the atmosphere and preventing the gas from leaving the liquid, thus reducing the hydroxyl ion concentration. To achieve this, all gas-tight culture flasks were charged with a 5% CO_2 in

air mixture, introduced at a low flow rate through a sterilised Pasteur pipette plugged with non-absorbent cotton wool. Cell culture dishes were placed in incubation boxes which were flushed with 150ml of CO_2 from a metered supply, again through a plugged Pasteur pipette. The boxes were then sealed with gas-tight tape. When only a few plates were put into a particular box an open 50ml beaker of sterile water was included to prevent excessive evaporation of the culture medium.

2.6. Cell Culture Methods.

2.6.1. Maintenance of Cell lines.

The Chinese hamster lines used in these studies are all capable of growth attached to glass surfaces. Stock cultures were routinely maintained in Hams F10 medium in sterile 150ml clear, soda-glass 'medical' flat bottles at 37°C. Cultures of mouse C3H $10T^1/_2$ cells were maintained in $75cm^2$ T/C plastic flasks (Sterilin) as their ability to attach and grow on glass was poor. Under these conditions cells grew as a monolayer on the bottom of each horizontally placed vessel.

Cultures were not allowed to reach a point were cells were released from a confluent monolayer, since this can lead to an increased heterogeneity within the cell population. The routine sub-culture protocol, designed to keep cells in a state of exponential growth (4.3.1), never allowed the culture to reach complete confluence (2.6.4).

2.6.2. Preparation of Cell Suspensions from Monolayer Cultures.

The essential procedure in the maintenance of cells in culture is sub-culture, a process involving the transfer of cells

from one culture vessel to another. For monolayer cultures attached to glass a suspension of cells must first be obtained. Once in suspension, cells can also be greatly diluted and plated allowing colonies to arise from single cells, a procedure fundamental to many experiments.

Prior to sub-culturing, cells in glass bottles were examined by naked eye. For routine sub-culture the medium was required to be clear, with no floating cellular debris, and not unduly acidic or basic as indicated by the phenol red component (orange through red to purple; acid to alkali). Cellular growth of more than 50% confluency was acceptable for experimental use. Where cultures were grown in plastic T/C flasks examination of cell growth under phase contrast microscope was possible. For these cultures cells had to have a normal appearance i.e. either epithelioid or fibroblastoid, depending on the cell type, with no refractile granules in the cytoplasm. If a particular culture failed to fulfil these requirements it was discarded.

The medium was removed from the culture and the monolayer rinsed with 5ml trypsin solution (2.3.3.). This was discarded and replaced with 1ml of fresh trypsin solution. The culture was incubated for either 5 min (CHO-K1 and C3H $10T^1/_2$ cells) or 3 min (V79-379A cells) at 37°C and then agitated to release the cells from the monolayer. The cell suspension was aspirated with a sterile Pasteur pipette, added to 5ml of serum-containing medium in a T/C tube, and sedimented by centrifugation. Although the Ca^{2+} and Mg^{2+} present in culture medium and serum provide some protection against the deleterious effects of the enzyme, re-suspension of the cells in typsin-free medium reduces its carry-over into fresh cultures.

The same method was used for the preparartion of cell suspensions for all cell lines employed during this study, although the composition of trypsin solution was varied (2.3.3.).

2.6.3. Determination of Cell Density.

This was achieved by haemocytometer count, a method which also allows the visual examination of the cells prior to experimentation. Cells were thoroughly mixed before a small volume was withdrawn from just below the surface of the suspension, and introduced into the haemocytometer chamber. Each chamber is divided into nine large squares by triple white lines. The four corner squares are divided into 16 squares. A total count was performed on eight large squares of the haemocytometer grid, (i.e. double chambered haemocytometer, 2.1.), under a phase contrast microscope. If cell clumping was observed, the count was disregarded, and the suspension aspirated to break up the clumps and a new sample taken. If the cell density was greater than 150 per large square, at which point counting becomes impeded by crowding, the suspension was further diluted and a repeat count was made. Conversely, if a very low cell density (<25 per large square) was observed, the cells were concentrated by centrifugation (1000 rpm for 5 min, Beckman TJ-6) and resampled.

The large squares each have an area of $1mm^2$. When the coverslip is pressed down over the grid , so that interferance patterns appear, the depth of the chamber is 0.1mm. The total volume over each large square is therefore:

$1 \times 1 \times 0.1 = 0.1mm^3 = 0.0001cm^3 = 10^{-4}$ ml

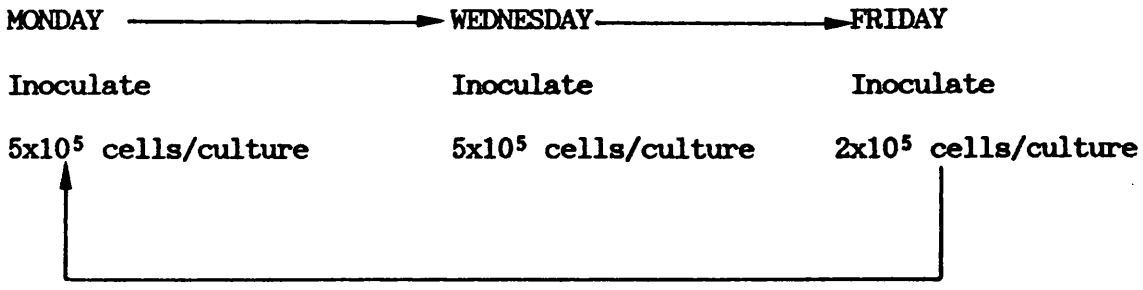
Hence total cell count per ml i.e. cell density, is given by:

Cell $ml^{-1} = 10^4 n$, where n is the average number of cells per large

square.

2.6.4. Sub-culture Routine.

Cells were sub-cultured, at low inoculation densities, at least three times a week to maintain cultures in a state of active growth. The following protocol was observed:



Aliquots of counted cell suspensions were added to 15ml volumes of culture medium, pre-warmed to 37.5°C, in standard 150ml 'medical' flat bottles and the cells evenly distributed by gentle agitation. Culture bottles were then purged with 5% CO_2 in air for 10-15 secs, capped tightly and returned to the incubator. Bottles were incubated flat to present the maximum surface area for growth. Photo-products, cytotoxic to mammalian cells, are known to be formed from certain media components (Wang *et al.*, 1974), therefore cultures were incubated in the dark. When a culture was required for use 3 days later $2x10^5$ cells were inoculated, and for use 2 days later, $5x10^5$ cells were inoculated.

2.6.5. Cell Storage.

Cells were routinely stored frozen in liquid N_2, or its overlying vapour, in the presence of a cryoprotectant. Either 5% DMSO v/v or 8% v/v glycerol were used as cryoprotectants. No

differences to the subsequent recovery of cells was observed with either compound.

Cells for storage were required to be in an actively growing state and hence a cell suspension was prepared from a culture ready for routine sub-culture. The cell density was determined and the suspension adjusted to a final cell density of $5x10^5$ cell ml^{-1} in medium containing the required volume of cryoprotectant. After thorough mixing, 1ml volumes were immediately distributed into 2ml screw-capped polypropylene ampoules. Batches of 8 ampoules were cooled at 1°C min^{-1} in the freezing unit of an LR-33-10 liquid N_2 freezer, to below -70°C, and then rapidly transfered to a liquid N_2 freezer for long term storage. A few days after every freezing operation a single ampoule was removed from storage. If the cells could be recovered to normal growth the freezing procedure was considered to be successful.

2.6.6. Recovery of Cells from Storage.

After removal of ampoules from the liquid N_2 refrigerator, the contents were rapidly thawed at 37°C in a water bath and the ampoule swabbed with 70% ethanol. The cells were transfered aseptically by sterile Pasteur pipette to 14ml of pre-warmed culture medium and evenly distributed by gentle agitation. The culture bottle was purged with 5% CO_2 in air and incubated at 37.5°C. After 2-3 hours the cells had usually attached to the glass surface and within 2-3 days had grown sufficiently to be passaged and subsequently handled as a routine stock culture.

2.6.7. Dilution Plating.

The plating of a small number of single cells into T/C

petri dishes, and their subsequent growth into macroscopic colonies, was fundamental to most experiments performed. This single-cell plating technique allows the determination of the plating (cloning) efficiency i.e. the proportion of individual cells in a given population capable of indefinite reproduction, and also the isolation of clonal populations (5.3.2.).

Although the dilution plating procedure varies with the nature of the particular experiment the following protocol is the basis for all the dilution plating procedures. 4.9ml aliquots of culture medium in 50mm T/C peri dishes were equilibrated at 37.5°C in a humidified 5% CO_2 in air atmosphere, in plastic incubation boxes. Attachment of cells can occur rapidly at 37.5°C (Ham and Puck, 1963) therefore to prevent cell loss, all other media and solution were used at room temperature. A cell suspension was prepared and the density determined. The counted cell suspension was serially diluted to a final cell density of 10^3 to $2x10^4$ viable cells ml^{-1} (dependent on the number of cells to be plated in a 0.1ml volume). For each step of the dilution procedure 0.5ml of the cell suspension was diluted with 4.5ml medium without FCS in a T/C tube then whirlimixed for 2 seconds. Mammalian cells tend to settle rapidly from suspension because of their large size and therefore to maintain a homogeneous cell suspension the dilution procedure was performed as quickly as possible and with frequent mixing. Replicate 0.1ml volumes of the final dilution were added to the equilibrated T/C dishes and the cells evenly distributed by gentle circular agitation. Inoculated dishes were replaced in the incubation boxes and with a 5% CO_2 in air atmosphere. The boxes were sealed with gas-tight tape and incubated in darkness at 37.5°C for 7-8 days. During incubation the dishes were left

undisturbed to prevent the formation of erroneous satellite colonies.

2.6.8. Staining.

To determine the plating efficiency of the cells, the medium was carefully poured fom the plates and then each flooded wtih 5% methylene blue in 50% v/v methanol. After 30 minutes the dishes were rinsed under running tap water and allowed to dry. Stained colonies were scored by naked eye against a white background, with aggregates containing 50 or more cells qualifying as a surviving colony. Colonies of marginal size were examined under a binocular dissecting microscope to confirm that they fulfilled this criterion. For all experiments, plates were coded and randomised prior to counting i.e. scored 'blind'.

CHAPTER 3. DETERMINATION OF AN ADAPTIVE PRE-TREATMENT PROTOCOL.

3.1. Introduction.

As reported in Chapter 1.4. a large variety of cell types have been used to characterise the mammalian adaptive response, each study having an unique combination of adaptive pre-treatment dose and pre-treatment protocol. It was important, therefore, to establish a sub-lethal MNNG concentration and a pre-treatment protocol applicable to both CHO-K1 and V79-379A cell lines prior to the commencement of this study. Frosina *et al.*, (1984) used a total of 49 protocol combinations and a number of mutagen concentrations during their work with V79 cells. For this study, however, two protocols were used which have previously been reported to be effective in the induction of an adaptive response, during work with V79 (Kaina, 1982) and CHO (Samson and Schwartz, 1980) cell lines, respectively.

In the following sections experiments to determine a sub-lethal MNNG concentration for each cell line are outlined. Two different adaptive pre-treatment schedules are reported. The effect of adaptive pre-treatment is assessed by the change in the MNNG dose-responses of the cells.

3.2. Toxic reponse tests to MNNG.

One of the major aspects of the bacterial adaptive response, (1.2.), is the enchancement of cell survival, to further mutagen challenge, following adaptive pre-treatment. It was necessary therefore to determine the dose responses (survival or kill curves) to MNNG for each cell line studied. The results obtained from this work would serve two purposes namely, (a) as a reference for comparison against the dose-responses observed in future adaptive experiments and (b) as an indication of mutagen doses likely to yield meaningful results in the mutational and sister chromatid exchange analyses detailed in chapters 4 and 7. For a given mammalian cell line the dose range over which a mutagenic agent produces lethality is usually that over which it produces significant mutation and sister chromatid exchanges (Kao and Puck, 1969; Carver *et al.*, 1979; Latt *et al.*, 1975).

Survival curves for MNNG-induced cytotoxicty were therefore constructed for both CHO-K1 and V79-379A cell lines.

3.2.1. The cytotoxic action of MNNG on CHO-K1 cells.

$5x10^5$ CHO-K1 cells were inoculated into 150 ml culture bottles containing 15 ml Hams F10 + 5% FCS, gassed with 5% CO_2 in air and incubated (2.6.4.). After two days growth, a cell suspension was prepared (2.6.2.) and the cell density determined (2.6.3.). Aliquots of this cell suspension were serially diluted to final cell densities of 10^3 - $8x10^4$ cell ml^{-1} (2.6.7.). Depending on the expected lethality, replicate 0.1 ml volumes of these suspension were dispensed into 50mm T/C dishes containing 4.9 ml Hams F10 + 5% FCS to give 100-8000 cells/plate. At each MNNG dose level triplicate petri-dishes were seeded. Initially the

number of cells per plate per dose level was calculated from previous reports using this cell line (Wilson, 1985). For example, if there was an expected cell survival of 1%, following a known MNNG dose administration, 6000 cells were seeded into each plate anticipating a final colony count of approximately 60 colonies per plate. During all survival estimations a final colony count of between 40 and 140 colonies per plate were aimed for, although this was not always achieved since control plating efficiencies ranged from 0.70 to 0.95. Using the data of Table 3.1. to illustrate this point, a 1% survival resulted from a dose administration of 0.2 $\mu g ml^{-1}$ MNNG, but, since the control plating efficiency in this particular experiment was 0.87, a mean count of 52 colonies per plate was observed rather than the 60 colonies, theoretically possible. Differences of control plating efficiencies may result from differences in culture condition between experiments e.g. slight differences in pH, gaseous atmosphere, length of time in culture etc.. When the likely cytotoxicity is uncertain, cells were plated at more than one density. The inoculated plates were incubated for 3 hours to allow cells to attach to the plates and also recover from trypsinisation. The test concentration of mutagen in vehicle was added in a 50 µl aliquot. After 2 hours mutagen exposure the medium was replaced with pre-warmed, fresh Hams F10 + 5% FCS. The plates were re-incubated and left undisturbed for 7 days, then stained and scored 'blind' (2.6.8.). Percentage survival was calculated as:

$$R_i = Y_i / Y_o \times 100$$

where;

R_i = Percentage cell survival.

Table 3.1. Data illustrating the survival of CHO-K1 cells grown in Hams F10 + 5% FCS, treated with MNNG.

Dose MNNG μgml^{-1}.	Cells plated	Colonies/plate	Mean	Plating efficiency	% Survival
0	100	87,90,89	87	0.87	100
0.02	200	50,44,62	52	0.26	30
0.03	500	55,63,62	60	0.12	14
0.04	1000	63,68,58	63	0.063	7.2
0.1	2000	52,56,60	56	0.028	3.2
0.2	6000	46,57,53	52	0.0087	1.0
0.3	8000	30,37,35	34	0.0042	0.48

Y_i = The plating efficiency after treatment i (mutagen).

Y_o = The plating efficiency after treatment o (vehicle).

Plating efficiency = Ratio of colonies formed to cells inoculated.

Data for the dose-response curve is presented numerically on Table 3.1. and graphically in Fig.3.1. as a plot of percentage survival, on a log scale, against MNNG dose. This curve is bi-phasic showing an immediate exponential reducton in cell survival with mutagen doses up to 0.05 μgml^{-1} MNNG. At MNNG concentrations between 0.05 μgml^{-1} and 0.3 μgml^{-1} there is a further exponential reduction in cell survival but the rate of reduction is slower. Extrapolation of the latter portion of this curve shows an intercept with the y-axis at a value of 6% survival, which represents the percentage of the resistant cell sub-population.

The D_{37} value, defined as the dose required to kill 63% of the cell population (37% survival) is considered to be a valid measure of cytotoxic potency (Carver *et al.*, 1979). Estimation of a D_{37} value from Figure 3.1. would be unsatisfactory since this respresents the results of a single experiment. For a more accurate measure of the D_{37} value of MNNG on CHO-K1 cells, and as a demonstration of the reproducibility of this technique, six dose-survivor curves were constructed. For each dose level triplicate plates were seeded with CHO-K1 cells resulting in a total of 18 survival determinations. These data are presented graphically in Figure 3.2. which shows a plot of the mean percentage survival ± standard error, plotted on a log scale, against MNNG concentration. From this figure the inflection point of the curve can be calculated as 0.05 μgml^{-1} MNNG at a survival level of 4%, i.e. the cross-over point of the extrapolated linear

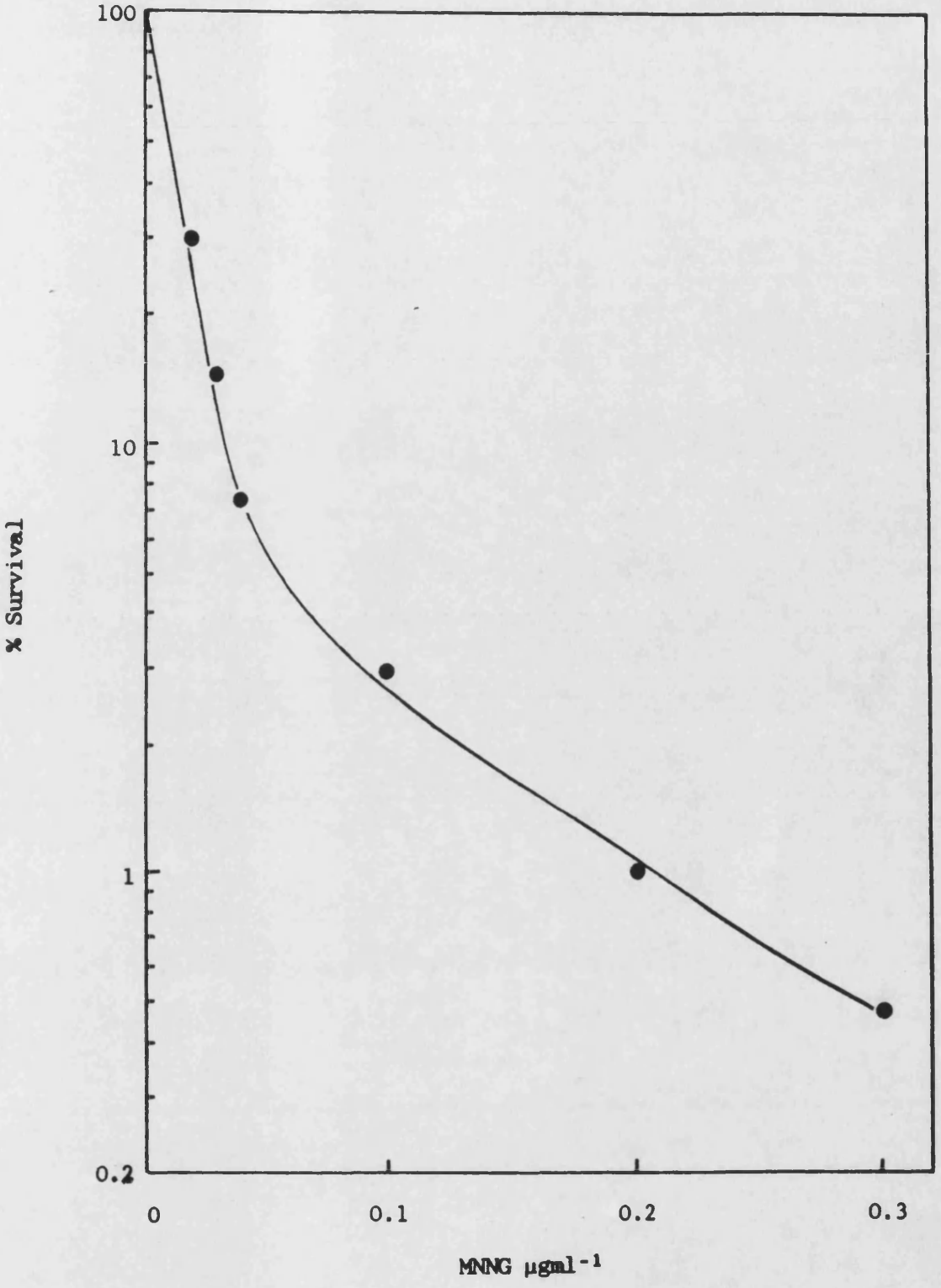


Figure 3.1. Dose-response curve for CHO-K1 cells grown in Hams F10 + 5% FCS, treated with MNNG.

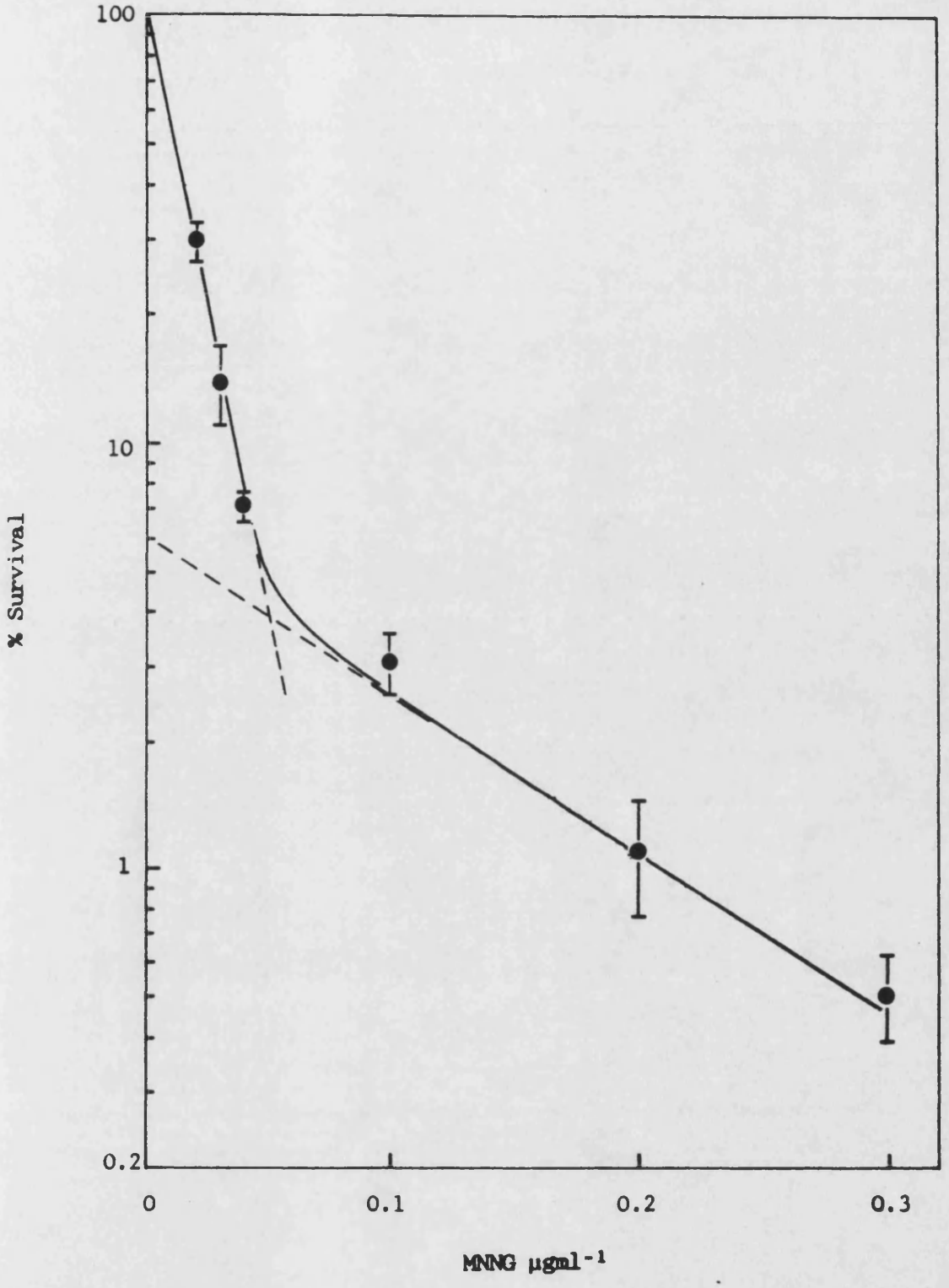


Figure 3.2. Reproducibility of the dose-response curve for CHO-K1 cells grown in Hams F10 + 5% FCS, treated with MNNG. In this and subsequent plots each point is the mean value from n replicate experiments ± the standard error i.e. in this instance n=6 ± S.E.

exponential portions. The D_{37} value was calculated from this Figure and was found to be 0.021 μgml^{-1} MNNG. This value is comparable to those obtained by Dewdney (1982) (0.035 μgml^{-1}) and Wilson, (1985) (0.021 μgml^{-1}), during their studies on CHO-K1 cells. Barranco and Humphrey, (1971) working on CHO cells, reported a similar bi-phasic response to MNNG, but with the inflection point arising at 3% survival and at a dose level of 0.5 μgml^{-1}. This value, and the entire survivor curve, differ by a factor of 10 from the data shown in Figs.3.1. and 3.2. The CHO-K1 cells used in this study are considerably more sensitive to the cytotoxic effects of MNNG than are their parental strain CHO, a fact confirmed by Dewdney, (1982) and Wilson, (1985).

3.2.2. The cytotoxic action of MNNG on V79-379A cells.

Dose-response curves for V79-379A cells treated were constructed using single plated cells. In preliminary studies by Wilson, (1985), MNNG concentrations used by other workers on V79 cells (Roberts *et al.*, 1971, Lankas *et al.*, 1977), were used but were found to be far too toxic towards V79-379A cells so the doses were reduced by a factor of 10. The MNNG concentrations eventually employed were identical to those used to produce the the CHO-K1 dose-survivor curve i.e. 0-0.3 μgml^{-1}.

The experimental protocol for V79-379A cells followed that used above for the determination of the CHO-K1 dose-response curve. (3.2.1.)

Survival data, obtained from six independent experiments, are shown in Figure 3.3. These are presented as mean percentage survival ± standard error, plotted on a log scale, against MNNG concentration. The D_{37} value for MNNG on V79-379A cells was

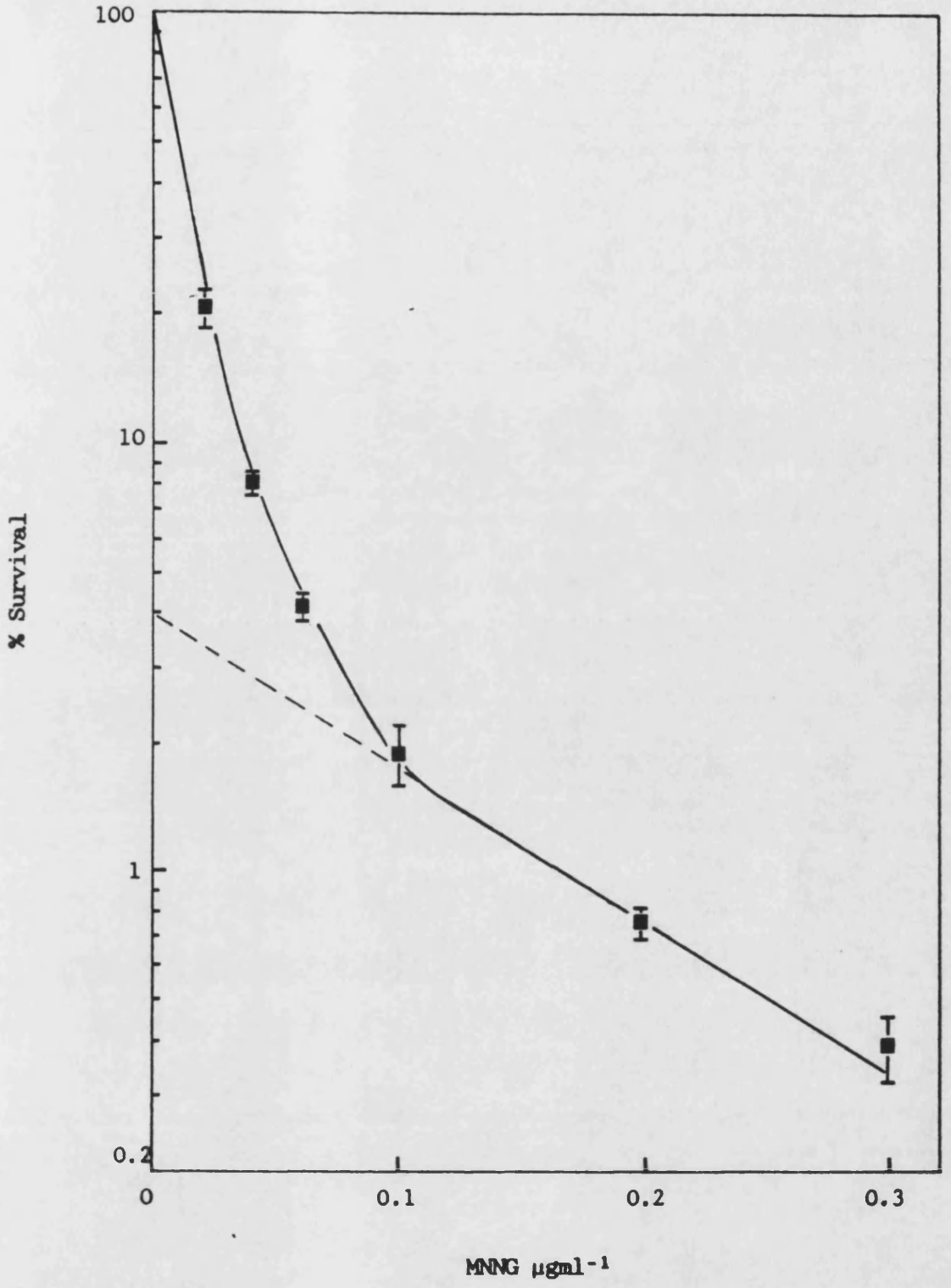


Figure 3.3. Reproducibility of the dose-response curve for V79-379A cells grown in Hams F10 + 5% FCS, treated with MNNG. (n=6 ± S.E.)

determined as 0.012 μgml^{-1}. As for CHO-K1 the V79-379A curve is bi-phasic but in contrast to the CHO-K1 curve, the inflection in the V79-379A curve occurs at an MNNG dose of 0.1 μgml^{-1} and a survival level of 2%.

3.3. Determination of a sub-lethal concentration of MNNG for the adaptive pre-treatment of CHO-K1 and V79-379A cells.

Before any adaptation experiments could be undertaken it was necessary to determine the adaptation dose. Essentially this dose has to be sub-lethal and sub-clastogenic i.e. causes no cytotoxicity or alteration to the chromosomal integrity, Kaina, (1982).

3.3.1. Toxic response tests on CHO-K1 and V79-379A cells exposed to MNNG concentrations in the range 0-0.02 μgml^{-1}.

CHO-K1 and V79-379A cultures were set up in two 150 ml culture bottles containing 15 ml Hams F10 + 5% FCS, gassed with 5% CO_2 in air and incubated (2.6.4.). After 46-48 hours a cell suspension was prepared for each cell line, counted and serially diluted to a density of 10^3 cell ml^{-1}. To 4.9 ml of pre-warmed Hams F10 + 5% FCS in 50 mm T/C dishes. 0.1 ml aliquots were dispersed and agitated to distribute the cells evenly. After 3 hours incubation in a 5% CO_2 in air atmosphere, the cells were exposed to the mutagen in 50 µl vehicle, the control plates receiving vehicle alone. Triplicate plates were set up for each dose. The cells were incubated for a further 2 hours when the medium was replaced with 5 ml of pre-warmed Hams F10 + 5% FCS. After 7 days incubation the medium was poured away and the colonies stained and scored.

The results of these experiments are presented as dose response plots in Figure 3.4. (a) and (b). Although the profiles are slightly different for the two lines, the critical or threshold MNNG concentration seems to be similar in both instances i.e. 0.01 μgml^{-1}, the point at which a decrease in cell survival begins. Therefore it was decided from this evidence that the adaptive pre-treatment dose, to be used in future experiments on both cell lines, would be 0.01 μgml^{-1}.

Fig.3.4. (a) and (b) also show that there is an initial shoulder to the dose-response curve for each cell line, a feature that was not apparent in Figs.3.2. and 3.3.

3.4. Determination of an adaptive pre-treatment protocol for CHO-K1 and V79-379A cells.

The criteria governing the choice of an adaptation pre-treatment protocol were; a) the protocol should previously have been shown to produce an adaptive effect in one or both cell lines, and b) the chosen protocol must show an effect in both CHO-K1 and V79-379A cell lines used in this study.

3.4.1. The effect of a single sub-lethal MNNG dose followed by MNNG challenge 6 hours later (Protocol 1) : toxic resposes of CHO-K1 and V79-379A cells.

The protocol used by Kaina, (1982) was followed. This involved the seeding of cells into T/C dishes and immediately treating them with a single adaptation dose of 0.01 μgml^{-1} MNNG. MNNG challenge was administered after 6 hours incubation producing a dose-response curve.

Two days prior to the experiment CHO-K1 and V79-379A

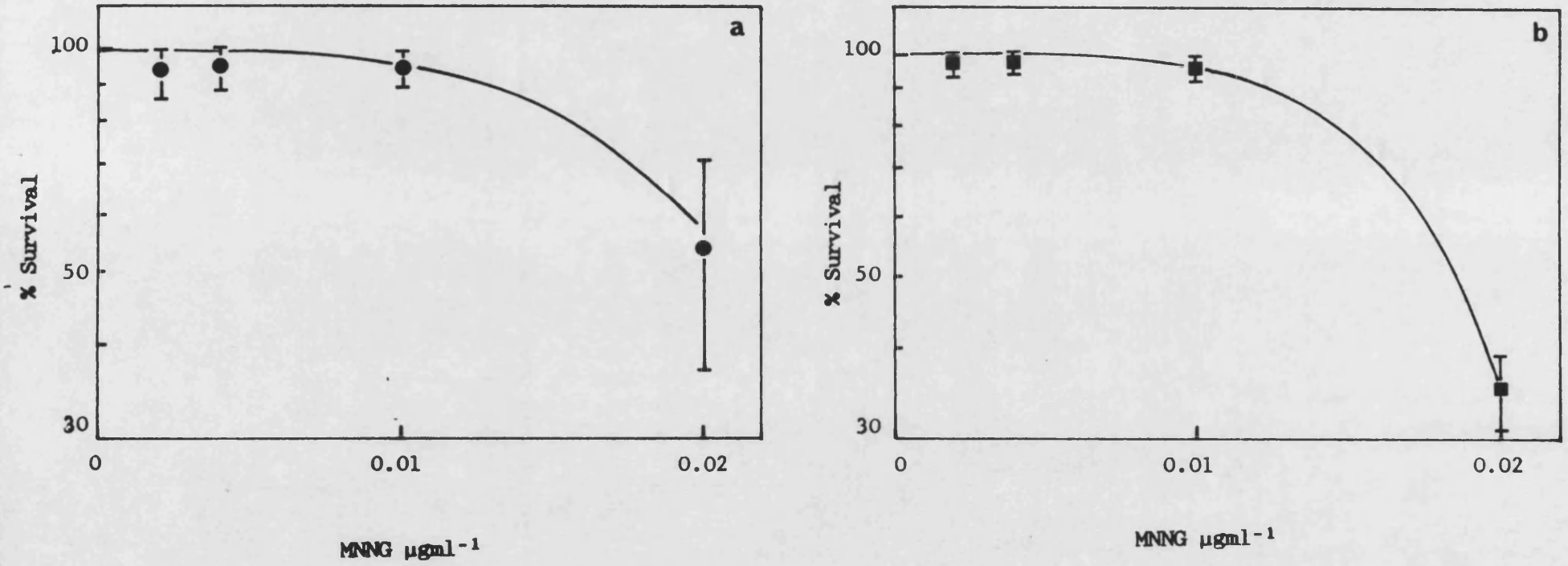


Figure 3.4. Dose-response curves for CHO-K1 cells, (a), and V79-379A cells, (b), grown in Hams F10 + 5% FCS, treated with MNNG in the dose range 0-0.2 μgml^{-1} . (n=3 ± S.E.)

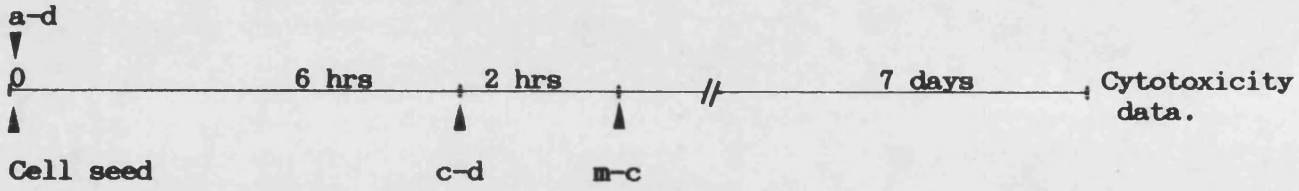
cultures were set up by inoculationg $5x10^5$ cells into two 150 ml culture bottles containing 15ml Hams F10 + 5% FCS, gassed with 5% CO_2 in air and incubated at 37°C. After 46-48 hours growth a cell suspension was prepared for each cell line, counted and serially diluted to densities between 10^3 and $8x10^4$ cells ml^{-1}. Depending on the expected cytotoxicity (3.2.1. and 3.2.2.) 100-8000 cells were plated into 50 mm T/C petri dishes containing pre-warmed Hams F10 + 5% FCS. Immediately after seeding the adaptive MNNG dose (0.01 $\mu g ml^{-1}$) was administered to each plate. Following a 6 hour incubation period, in a 5% CO_2 in air atmosphere, the medium was poured off and the plates rinsed with pre-warmed PBS (2.3.2.) and replaced with 5ml fresh pre-warmed Hams F10 + 5% FCS. Mutagen challenge doses up to 0.3 $\mu g ml^{-1}$ were now added and the cells incubated for 2 hours before another medium change was carried out. After 7 days incubation the cells were stained and scored 'blind' (Fig.3.5.). Control cells were exposed to the same procedure substituting vehicle for the pre-treatment dose.

The effect of this type of pre-treatment on both CHO-K1 and V79-379A cells is presented in Fig. 3.6. (a) and (b) respectively. The results of three independent experiments are shown.

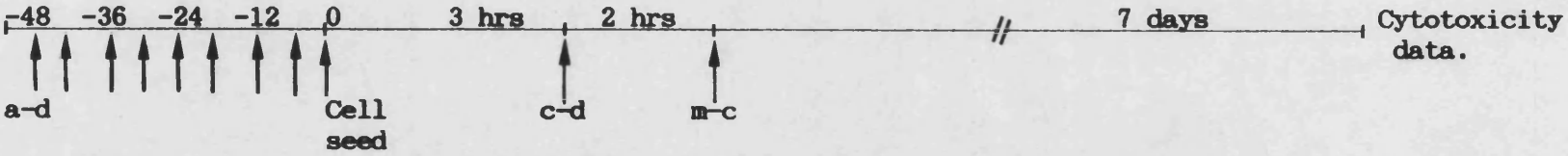
CHO-K1 cells exhibited no significant change in the dose response curve when pre-treated and control cells were compared. The D_{37} values were 0.06 $\mu g ml^{-1}$ in both instances. This value is higher than that of 0.021 $\mu g ml^{-1}$ observed in 3.2.1. for CHO-K1 cells. V79-379A cells, however, show a 2-fold enhancement of survival after pre-treatment on comparison of the D_{37} values for treated (0.021 $\mu g ml^{-1}$) and untreated control cells (0.011 $\mu g ml^{-1}$). This agrees with the findings of Kaina, (1982) who worked on V79-C10 cells and also found a 2-fold increase in cell survival on

Figure 3.5. Diagrammatic representation of adaptation protocols 1 and 2.

Protocol 1 (Kaina, 1982).



Protocol 2 (Samson and Schwartz, 1980).



a-d = MNNG adaptive dose (0.01 μg ml^{-1}).
c-d = MNNG challenge doses (0.02-0.3 μg ml^{-1}).
m-c = Medium change.

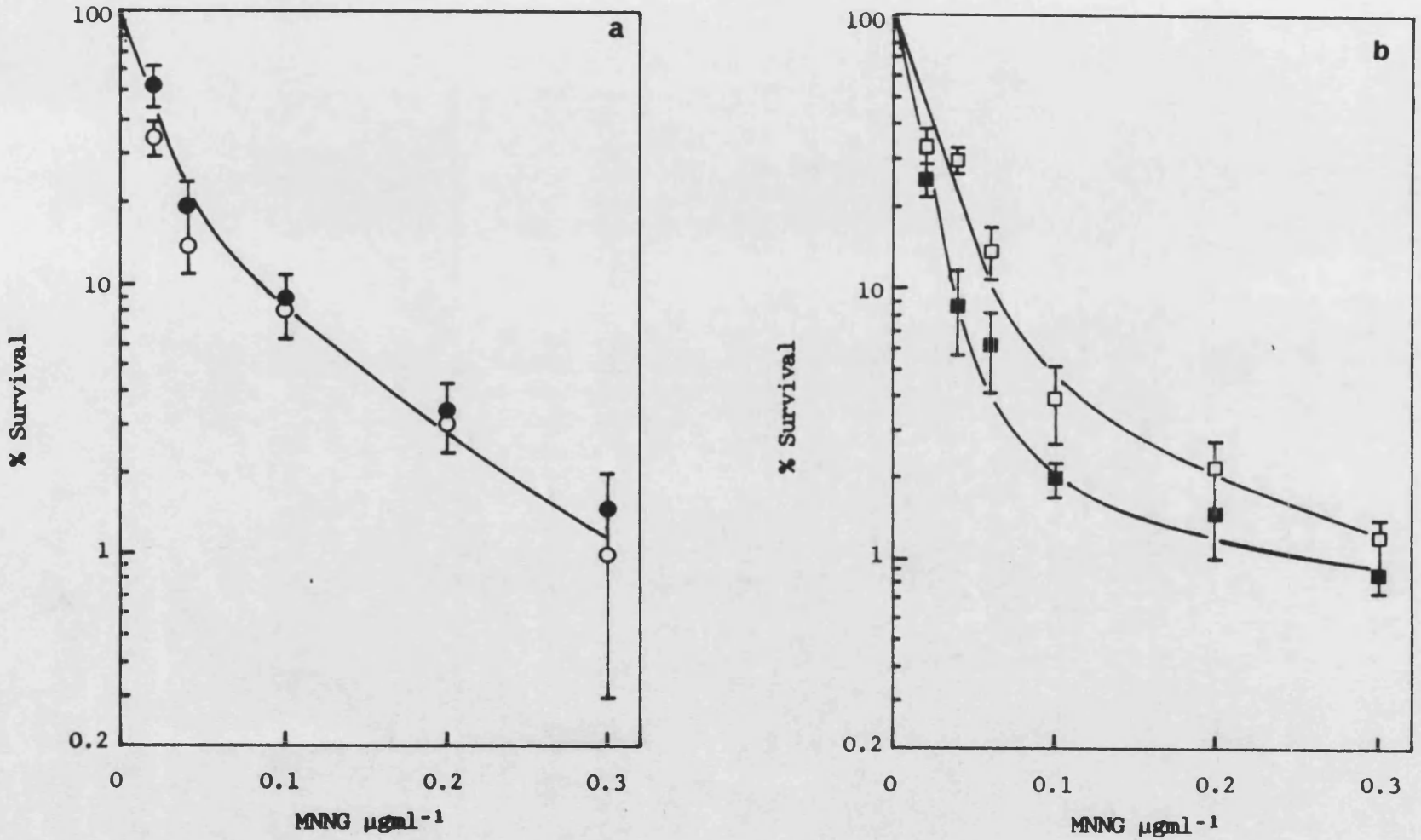


Figure 3.6. Dose-response curves for CHO-K1 cells (a) and V79-379A cells (b), grown in Hams F10 + 5% FCS, after pre-treatment with a single 0.01 $\mu g ml^{-1}$ MNNG dose followed by MNNG treatment 6 hours later (Protocol 1). Open and closed symbols represent pre-treated cells and control cells respectively. (n=3 ± S.E.)

challenge after a single pre-treatment dose.

3.4.2. The effect of eight single sub-lethal MNNG doses at six-hourly. intervals for 48 hours, followed by MNNG challenge (Protocol 2): toxic responses of CHO-K1 and V79-379A cells.

This protocol followed that used by Samson and Schwartz, (1980).

Two days prior to the experiment CHO-K1 and V79-379A cultures were set up by inoculating $5x10^5$ cells into two 150 ml culture bottles containing 15 ml Hams F10 + 5% FCS, incubation at 37.5°C in a 5% CO_2 in air atmosphere. After 46-48 hours growth a cell suspension was prepared and the cell density determined. Eight culture bottles were now set up each containing 15 ml Hams F10 + 5% FCS. To four of these, $2x10^5$ CHO-K1 cells were inoculated. To the other four, $2x10^5$ V79-379A cells were added. Within each set of four bottles two received 50 µl vehicle (H_2O) and two received the MNNG adaptive dose (0.01 $\mu g ml^{-1}$). This dose was designated to be at time t=-48 (Fig. 3.5.). After 6 hours the medium in each bottle was replaced by 15 ml fresh Hams F10 + 5% FCS containing either vehicle or the MNNG adaptive dose of 0.01 $\mu g ml^{-1}$. The bottles were gassed with 5% CO_2 in air and incubated for a further 6 hours. The procedure of media replacement and MNNG administration was repeated every 6 hour until a total of 8 pre-treatment doses had been given i.e. at t=-6. Six hours after the final dose (t=0) cell suspensions were prepared, the cells counted and serially diluted to cell densities between 10^3 and $8x10^4$ cells ml^{-1}. 0.1 ml aliquots of cells were distributed into 50 mm T/C dishes containing 4.9 ml pre-warmed Hams F10 + 5% FCS to give between 100 and 8000 cells/plate. The expected cytotoxicity

of pre-treated cells was uncertain so for each challenge dose a range of cell densities were prepared. After 3 hours incubation the cells were exposed to MNNG challenge doses (up to 0.3 μgml^{-1}) and left a further 2 hours before a change of medium was performed for each plate. The cells were allowed to grow in the incubator for 7-9 days (see 4.3.2.), stained and scored 'blind'.

The results of three independent experiments for both CHO-K1 and V79-379A cells are presented in Figs. 3.7. and 3.8. respectively, presented as mean percentage survival, plotted on a log scale, against MNNG concentration. It is observed that at each challenge dose pre-treated cells, in both lines, survived better than control cells. CHO-K1 cells exhibited a 3-fold difference in D_{37} value over control (0.014 to 0.042 μgml^{-1}) and V79-379A cells a 6.8-fold difference in the D_{37} value compared to control (0.012 to 0.082 μgml^{-1}). For both cell lines the bi-phasic dose-response observed in untreated cells is also observed for the pre-treated cells.

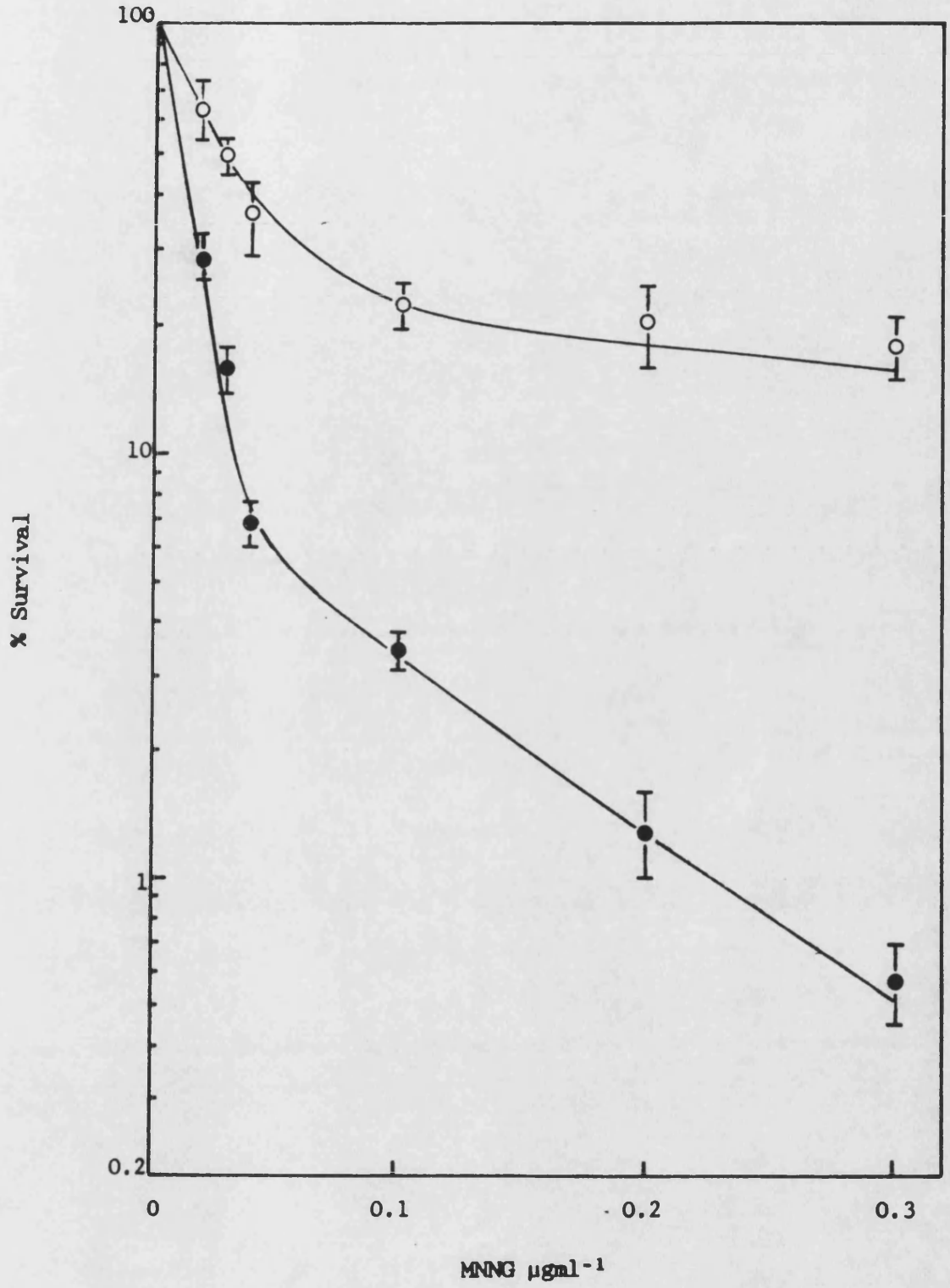


Figure 3.7. Dose-response curves for CHO-K1 cells grown in Hams F10 + 5% FCS, pre-treated with a 0.01 μgml^{-1} MNNG dose every six hours for 48 hours (Protocol 2), then treated with MNNG. Open and closed symbols represent pre-treated cells and control cells respectively. (n=3 ± S.E.)

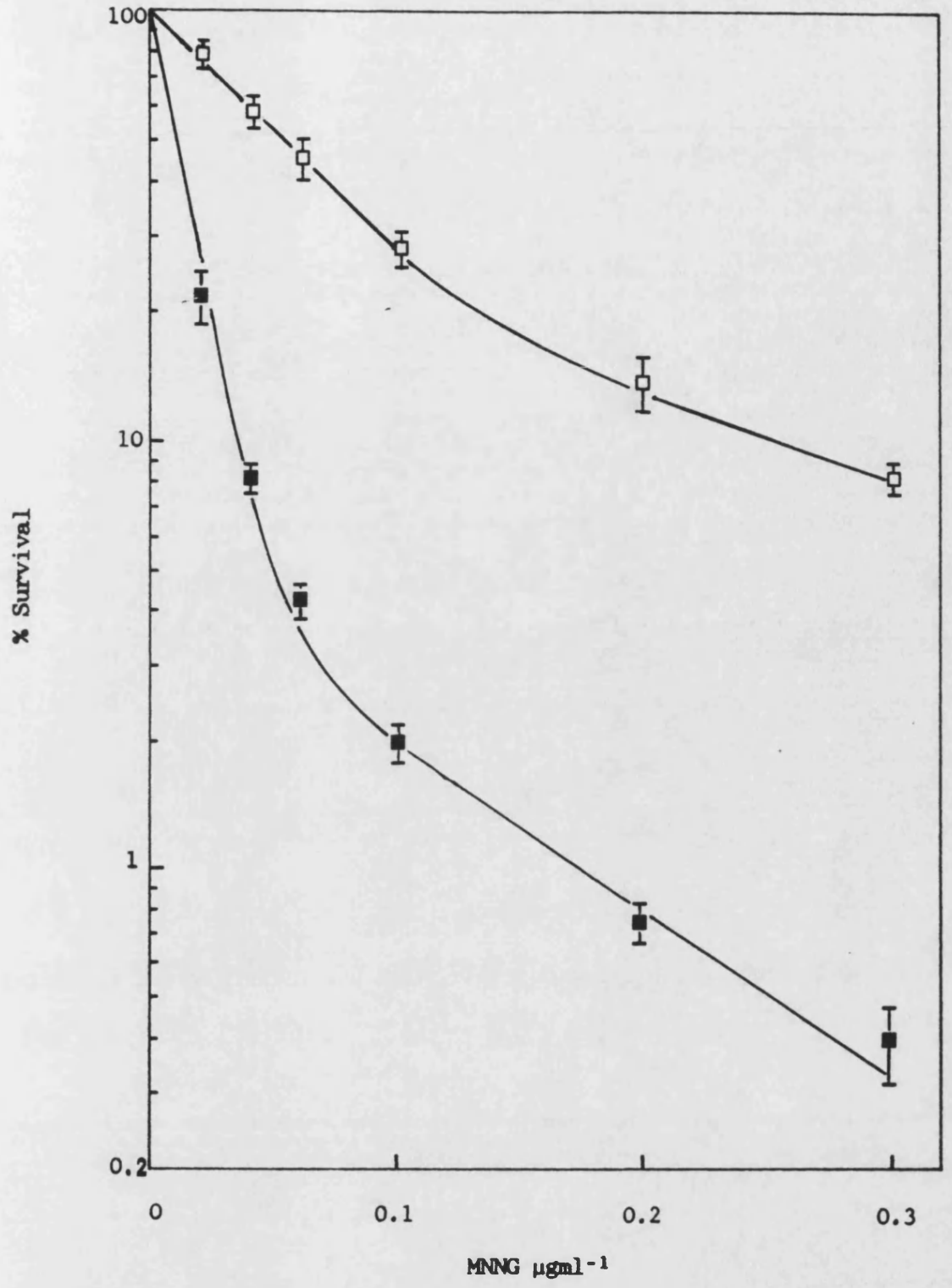


Figure 3.8. Dose-response curves for V79-379A cells grown in Hams F10 + 5% FCS, pre-treated with a 0.01 μgml^{-1} MNNG dose every six hours for 48 hours (Protocol 2), then treated with MNNG. (n=3 ± S.E.). Open and closed symbols represent pre-treated and control cells respectively.

3.5. Conclusions.

From the results reported in this chapter the following can be concluded:

1) MNNG, in the range 0.02 to 0.3 μgml^{-1}, is cytotoxic to both CHO-K1 and V79-379A cells in a bi-phasic, dose-dependent fashion (3.2.1. and 3.2.2.). D_{37} values derived are 0.021 μgml^{-1} and 0.012 μgml^{-1} respectively.
2) Below 0.01 μgml^{-1} MNNG is non-toxic (sub-lethal) to both cell lines.
3) CHO-K1 cells pre-treated by protocol 1 exhibited no change in survival when compared to untreated cells (3.4.1.).
4) V79-379A cells pre-treated by protocol 1, however, exhibited a 2-fold enhancement of survival over untreated cells on comparison of the D_{37} values, i.e. 0.021 μgml^{-1} to 0.011 μgml^{-1}.
5) Adaptive pre-treatment by protocol 2 resulted in a 3-fold enhancement of survival of CHO-K1 over control cells. D_{37} values for pre-treated and untreated controls were 0.042 and 0.014 μgml^{-1} respectively.
6) Adaptive pre-treatment by protocol 2 resulted in a 6.8-fold, enhancement of survival of V79-379A over control cells. D_{37} values were 0.082 and 0.012 μgml^{-1} for pre-treated and control respectively.
7) From these data it is apparent that protocol 1 i.e. a single pre-treatment dose, does not fulfil the two criteria outlined in 3.4.. Therefore it was decided that the pre-treatment protocol to be used in all future adaptation experiments would be that of Samson and Schwartz, (1980) using multiple pre-treatment doses and set out diagrammatically in Fig.3.5. as protocol 2.

These conclusions will be discussed in chapter 8.

CHAPTER 4. THE EFFECT OF ADAPTIVE PRE-TREATMENT ON CHO-K1 AND V79-379A CELLS.

4.1. Introduction.

Previous studies into the mammalian adaptive response have revealed that mutagen pre-treatment enhanced survival, but did not always reduce mutation frequencies on subsequent challenge. Two independent groups, Durrant *et al.*, (1981) and Frosina *et al.*, (1984), both working with V79 cells, observed an increased survival without the accompanying mutagenic adaptation. However, Kaina, (1982) and Laval and Laval, (1984), working on V79 and rat H_4 lines respectively, reported the presence of both lethal and mutagenic adaptation. In each case the mutation frequencies were measured by the induction of resistance to the purine analogue 6-thioguanine. It was important, therefore, to determine whether the enhanced cell survival observed in both CHO-K1 and V79-379A cells (3.4.2.) was accompanied by a similar enhancement of the mutagenic response. Since O^6-methyl-guanine is considered to be the most important pre-mutagenic lesion induced in DNA by methylating agents, (Chapter 1), an assay system which detects potential base-substitution mutations of this type was necessary. It was decided to assay mutations at the Na^+/K^+ ATPase locus using CHO and V79/ouabain assay systems instead of the relatively lengthy and expensive HGPRT, (6-thioguanine resistance), system used previously.

The effects of pre-treatment on the growth parameters of the two cell lines were also investigated.

Since the sensitivity of CHO cells to MNNG fluctuates throughout the cell cycle, (Barranco and Humphrey, 1971), a change in cell cycle distribution may alter the responses of the

overall population. Therefore CHO-K1 cells were synchronised and the effect of subsequent MNNG challenge, on the induced cytotoxicity and mutation to Oua^R, compared to the values obtained for asynchronous control and asynchronous pre-treated cells.

4.2. Mutation to Oua^R.

The active sodium and potassium ion transport system in mammalian cells is associated with the Na^+-K^+-Mg^{2+}-activated ATPase (Na^+/K^+ATPase)(E.C.3.6.1.3) located in the plasma membrane (Baker *et al.*, 1974). Resistance to the steroid compound ouabain (Oua), involves this enzyme. Ouabain binds to the alpha sub-unit of this enzyme specifically inhibiting its action and is ultimately cytotoxic. Resistance is probably the result of base-pair substitutuion occuring at only limited sites within the Na^+/K^+ATPase locus that affect the affinity (binding) of ouabain without destroying enzyme activity. Clones of both established and primary lines have been obtained, by single-step selection in Oua-containing culture medium, which have greatly increased resistance to Oua cytotoxicity when compared with wild-type cells (Arlett, 1977; Baker *et al.*, 1974; Davies and Parry, 1974; Cole and Arlett, 1976).

A number of observations have confirmed that the occurence of ouabain resistance (Oua^R) reflects a mutant genotype:

a) The ouabain resistance phenotype is stable , being reproducible after long intervals of growth in the absence of the selecting drug (Baker *et al.*, 1974).

b) Luria-Delbruck fluctuation analyses indicate that Oua^R cells arise randomly in wild-type (WT) populations of both mouse L-cells and CHO cells, as expected of spontaneous mutations

(Baker *et al.*, 1974).

c) Treatment of WT cell populations with EMS and other mutagens causes a substantial increase in the frequency of Oua^R cells (Cole and Arlett, 1976; Davies and Parry, 1974; Gupta and Siminovitch, 1980).

d) Hybrids of Oua^R and ouabain-sensitive (Oua^S) WT CHO cells show intermediate resistance between that of the two parental cell strains, establishing that Oua^R is a co-dominant trait (Baker *et al.*, 1974).

The nature of the mutations responsible for Oua^R are suggested by the classes of mutagens most effective at inducing Oua^R mutants. EMS and UV, agents capable of inducing base-pair substitutions, are effective inducers of such mutations (Baker *et al.*, 1974; Arlett, 1977). Oua^R cannot be induced by either ionizing radiation or the frameshift mutagen ICR 191, (Bradley *et al.*, 1981), indicating that some Na^+/K^+ATPase activity is vital for cell survival, and frameshift or other inactivating mutations which eliminate enzyme activity, result in cell death. Mutations to Oua^R are thereforelikely to arise from base-pair substitutions resulting in a single amino acid or subtle configuration alteration in the relevant gene product, be it Na^+/K^+ATPase itself or some adjacent membrane component.

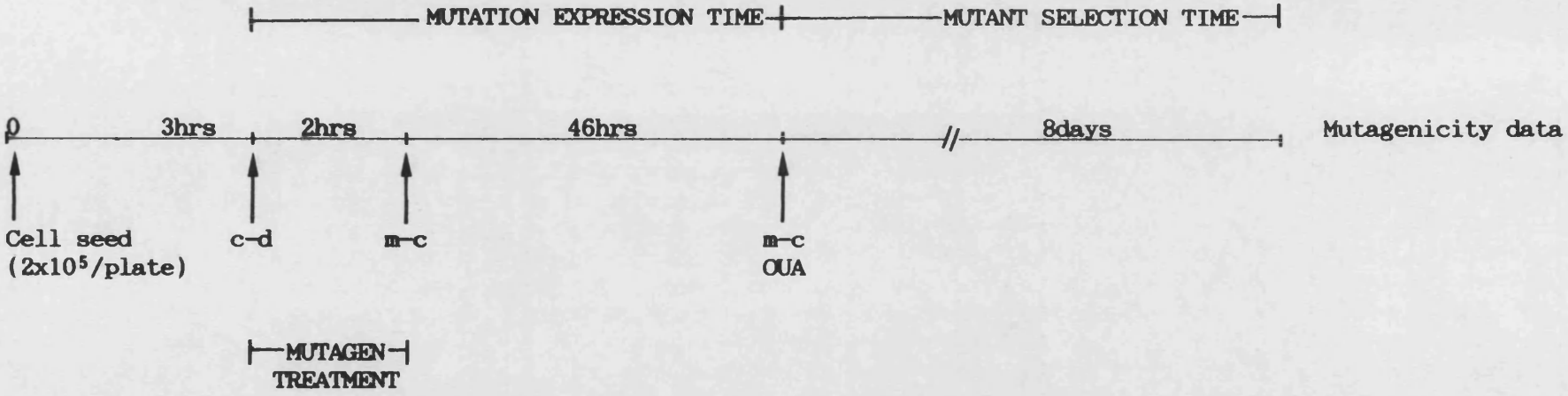
4.2.1. The MNNG-induced mutation of CHO-K1 cells to Oua^R.

Two days prior to the experiment duplicate cultures of CHO-K1 cells were set up by inoculating $5x10^5$ cells into 150ml culture bottles each containing 15ml Hams F10 + 5% FCS. After gassing with 5% CO_2 in air and incubating for 46-48 hours at 37.5°C, cell suspensions for each cell line were prepared and the

cell desity determined. These suspensions were used to inoculate $2x10^5$ cells into 90mm T/C dishes, (five for each mutagen dose i.e. $1x10^6$ cells in total) each containing Hams F10 + 5% FCS to 10ml (selection plates). Further dilution of these suspensions allowed survival plates to be set up in parallel as outlined in 3.2.1. For each mutagen dose level three petri-dishes were seeded with between 100 and 8000 cells per plate, dependent on the anticipated cytotoxicity. After 3 hours incubation, MNNG was added to both survival and selection plates in 50 µl of vehicle. Control plates received vehicle only i.e. DDH_2O. After a 2 hour mutagen treatment the culture medium in the was replaced with fresh, pre-warmed Hams F10 + 5% FCS and the plates re-incubated. Following a further 46 hour incubation, (mutant expression time. Fig.4.1), the medium in the selection plates was replaced with 9ml of Hams F10 + 5% FCS in conjunction with 1 ml of 10mM stock ouabain solution (2.4.2.) i.e. 1mM final Oua concentration. The plates were incubated, stained, coded and scored 'blind' after 7 days (survival plates) or 8 days (selection plates). A diagram summarising this experimental protocol is given in Fig.4.1. The optimum conditions for induction and detection of Oua^R mutants, in the CHO-K1 cell line, were validated by Dewdney, (1982). This system was also used on both CHO-K1 and V79-379A cells by Wilson, (1985) who showed that the optimum mutant expression time was between 47 and 49 hours before mutant selection.

Data obtained from experiments of this type can be expressed as mutation frequencies. These are calculated as the number of mutants, selected for by the selection medium, following mutagen treatment and expression, per 10^6 surviving cells. The observed mutation frequency is calculated as follows

Figure 4.1. Diagrammatic representation of the protocol used to assess the MNNG-induced mutation to Oua^R of CHO-K1 and V79-379A cells.



c-d : MNNG challenge doses (0.02-0.3 $ugml^{-1}$)
m-c : Medium change
OUA : Mutant selection medium - Hams F10 + 5% FCS + 1mM ouabain.

Observed mutation frequency =

$$\frac{\text{Total number of mutants scored} \times 10^6}{\text{Total number of cells plated into selection plates.} \times \text{Plating efficiency of cells in parallel survival plates.}}$$

.....Equation 4.1.

Table 4.1. presents cytotoxicity and mutation data obtained, for CHO-K1 cells, from a single experiment. This illustrates typical plate counts and each step in the calculation of the observed mutation frequency.

Computation of results.

Mutation frequencies (M.F.), in these studies, are expressed as mutants per 10^6 surviving cells. Using the data of Table 4.1., for an MNNG dose of 0.2 $\mu g ml^{-1}$, and working through using equation 4.1., the MNNG mutation frequency to Oua^R can be calculated.

Medium	Cells/plate	Colonies/plate	Mean	Total mutants
Non-selective	$4x10^3$	58,40,58	52	-
Selective	$2x10^5$	6,8,7,7,0	-	32

Plating efficiency = 52/4000 = 0.013

M.F. per survivor = $\frac{32 \times (10^6)}{(5x2x10^5) \times 0.013}$ = 2462 mutants per 10^6 survivors.

The data presented in Table 4.1. is shown in Fig.4.2. as a plot of induced mutation frequency to Oua^R ($Oua^R/10^6$ survivors), plotted on a log scale, against MNNG concentration. CHO-K1 cells exhibit a dose-dependent increase in mutation frequency when subjected to increasing concentrations of MNNG over the range 0.02

Table 4.1. Data illustrating the survival and mutation to Oua^R of CHO-K1 cells grown in Hams F10 + 5% FCS, treated with MNNG.

Survival data.

Dose MNNG $\mu g ml^{-1}$	Cells plated	Colonies/plate	Mean	Plating efficiency	% Survival
0	100	113,127,90	110	1.10	100
0.02	200	80,75,88	81	0.405	37
0.04	500	56,44,47	49	0.098	8.9
0.1	2000	65,70,70	68	0.034	3.1
0.2	4000	74,89,83	82	0.0205	1.86
0.3	6000	48,64,59	57	0.0095	0.86

Mutation data.

Dose MNNG $\mu g ml^{-1}$	Mutant colonies/plate	Total mutants	P.E. of survivors	Mutants/10^6 survivors*
0	0,0,0,1,0	1	1.10	0.91
0.02	3,3,4,9,4	23	0.405	57
0.04	12,3,6,8,4	33	0.098	337
0.1	6,6,6,6,4	28	0.034	824
0.2	10,16,12,17,8	63	0.0205	3073
0.3	12,10,8,13,14	57	0.0095	4515

* Calculated by Equation 4.1.

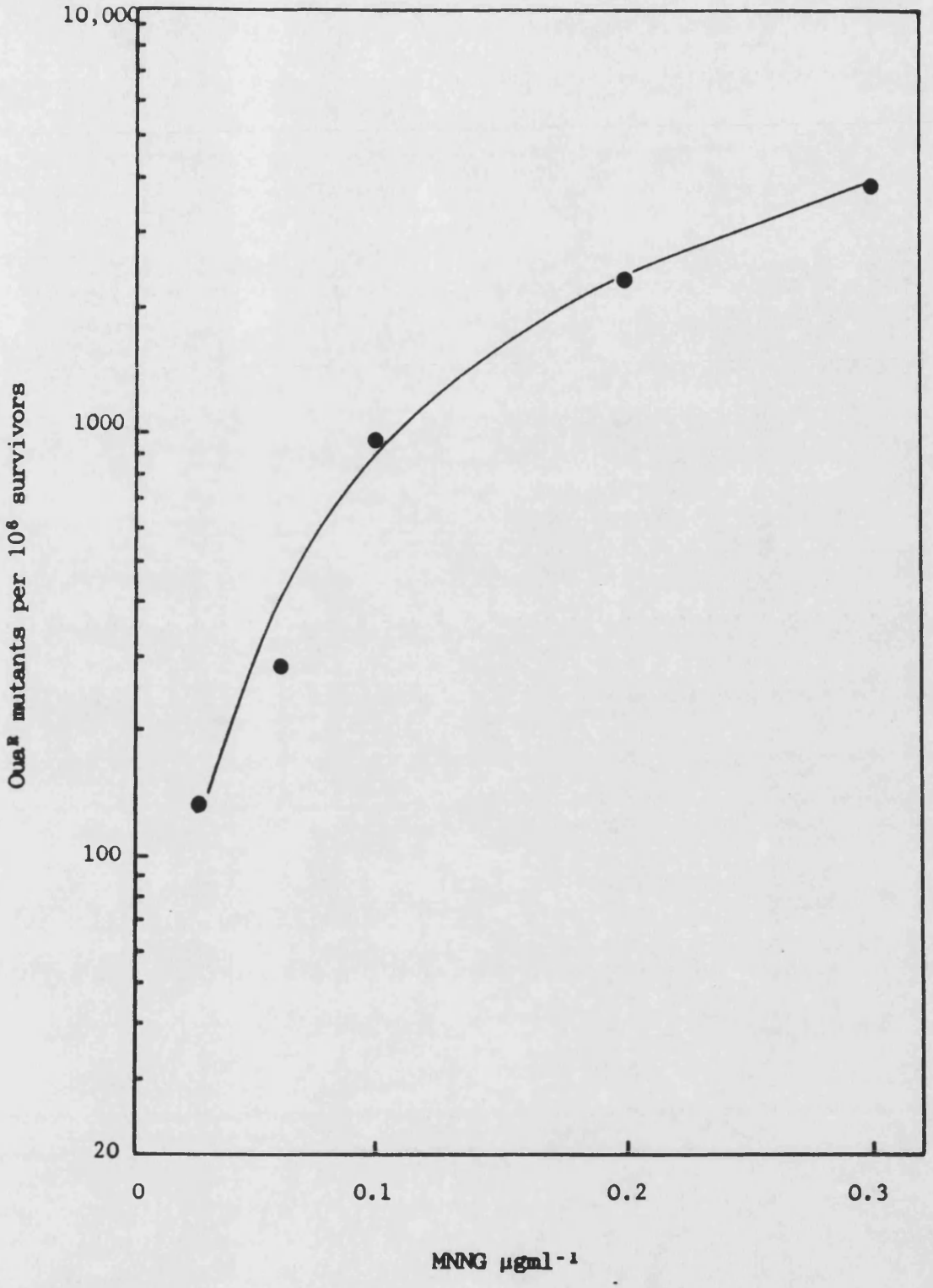


Figure 4.2. The dose-response curve of the MNNG-induced mutation frequency to Oua^R of CHO-K1 cells grown in Hams F10 + 5% FCS.

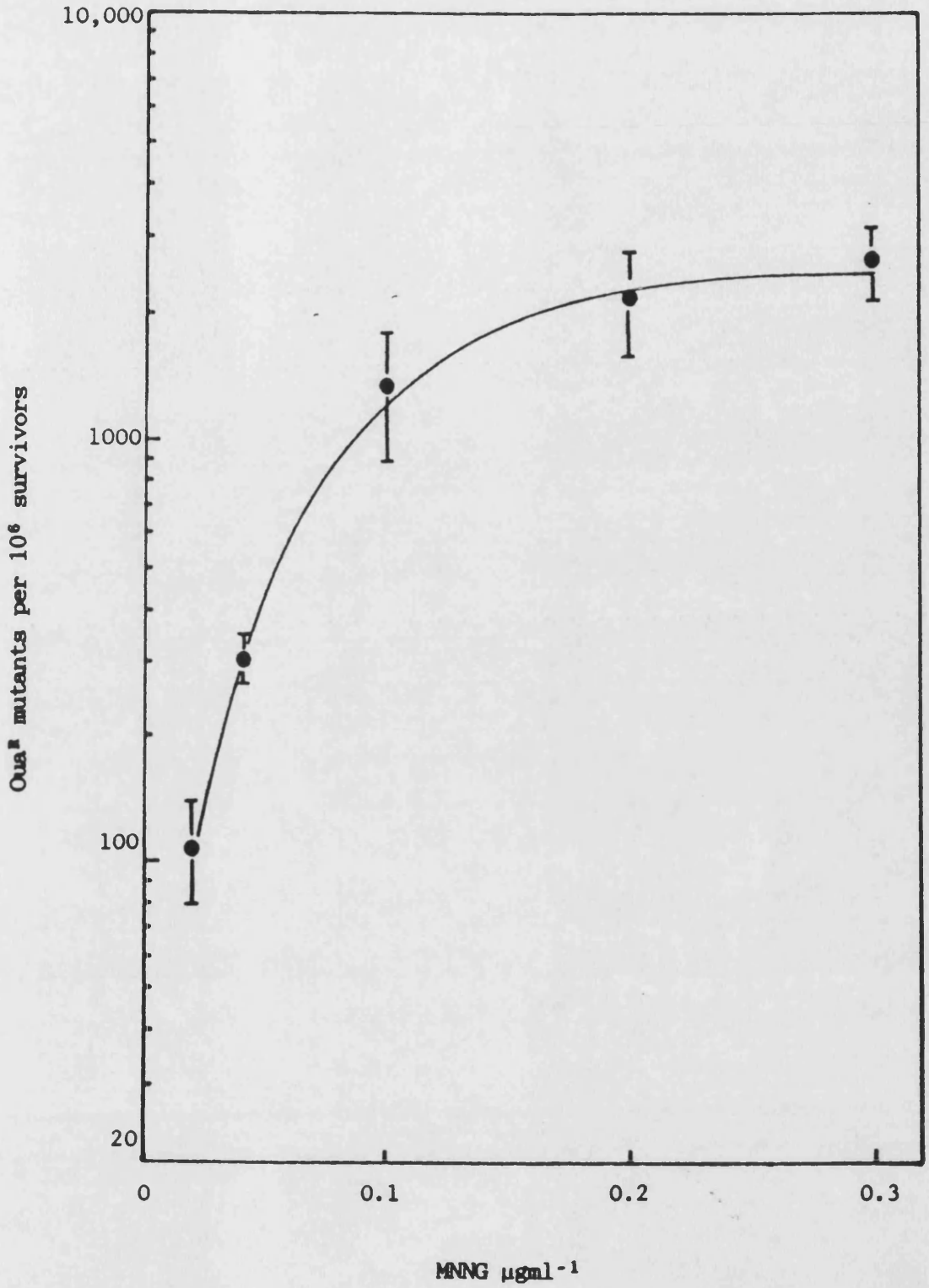


Figure 4.3. Reproducibility of the dose-response curve of the MNNG-induced mutation frequency to Oua^R of CHO-K1 cells grown in Hams F10 + 5% FCS. (n=6 ± S.E.)

μgml^{-1} to 0.3 μgml^{-1}. The response is curvilinear and hyperbolic. The reproducibility of this assay technique was demonstrated by performing six independent experiments the results of which are shown in Fig.4.3. expressed as mutation frequency, plotted on a log scale, against MNNG concentration.

4.2.2. The MNNG-induced mutation of V79-379A cells to Oua^R.

Dose-response curves of induced mutation to Oua^R for V79-379A cells were constructed using single plated cells. The experimental protocol for these cells followed that used above for determination of the CHO-K1 dose-response curve. The results of six independent experiments are shown in Fig.4.4. presented as mutation frequency, plotted on a log scale, against MNNG concentration.

Cells that are Oua^R, occur spontaneously with frequencies in the range <1-8 mutants/10^6 survivors (Arlett *et al.*, 1975; Lankas *et al.*, 1977; Lever and Seegmuller, 1976) and have been induced by a wide variety of chemical mutagens with frequencies in the range 5-4000 mutants/10^6 survivors (Arlett, 1975; Bradley *et al.*, 1981; Davies and Parry, 1974; Maleveille *et al.*, 1977). The results presented in Figs 4.2, 4.3. and 4.4. fall within these ranges. The spontaneous (control) mutation frequencies in these figures have not been plotted but were determined as 1.12×10^{-6} and 0.97×10^{-6} for CHO-K1 and V79-379A cells respectively

An alternative method of expressing the effect of MNNG on the mutation frequencies of either CHO-K1 or V79-379A cells, is to plot mutation against percentage survival, both on log scales. Munson and Goodhead, (1977) reported a linear relationship between log mutation frequency and log cell survival. Carver *et al.*,

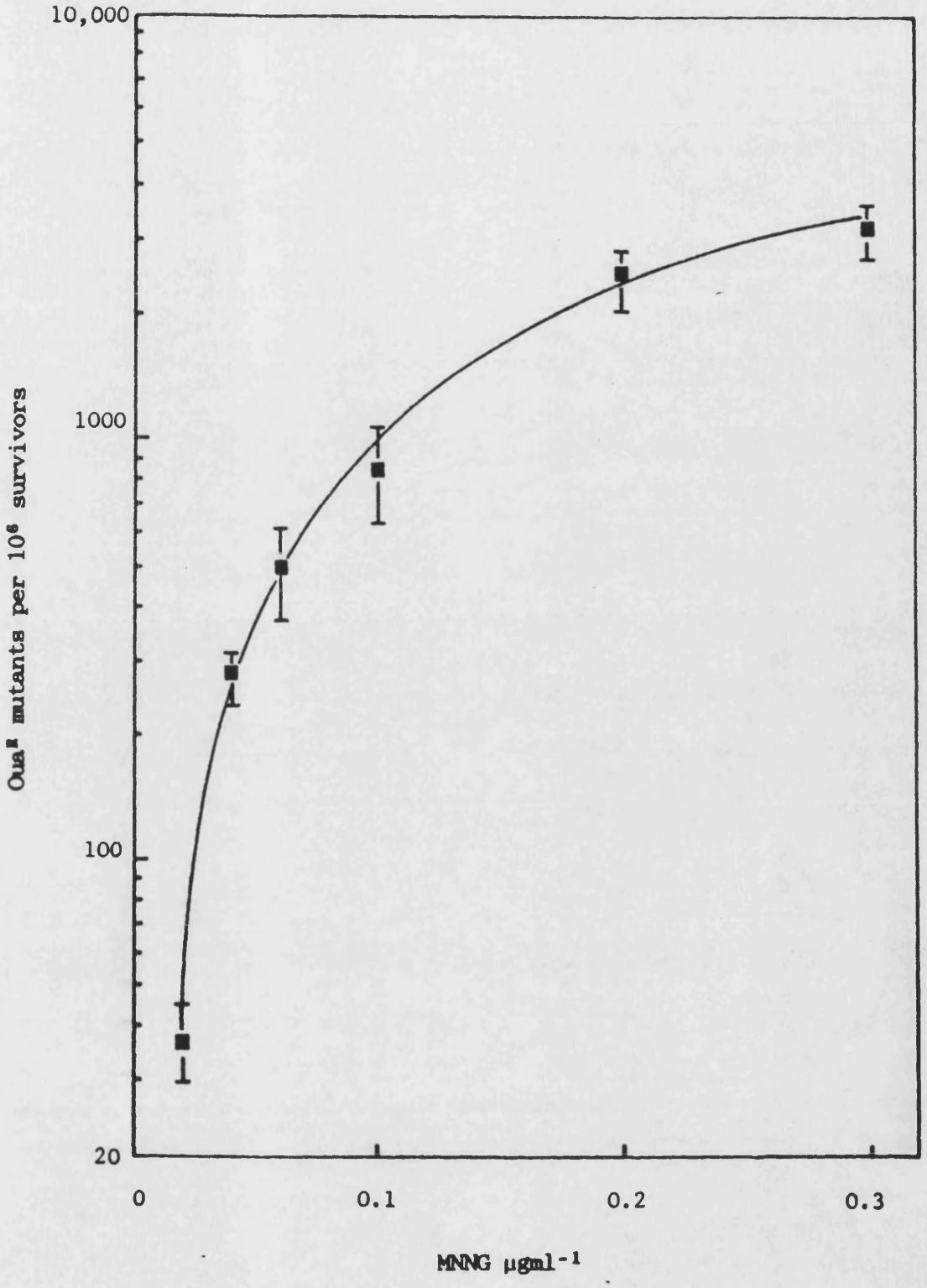
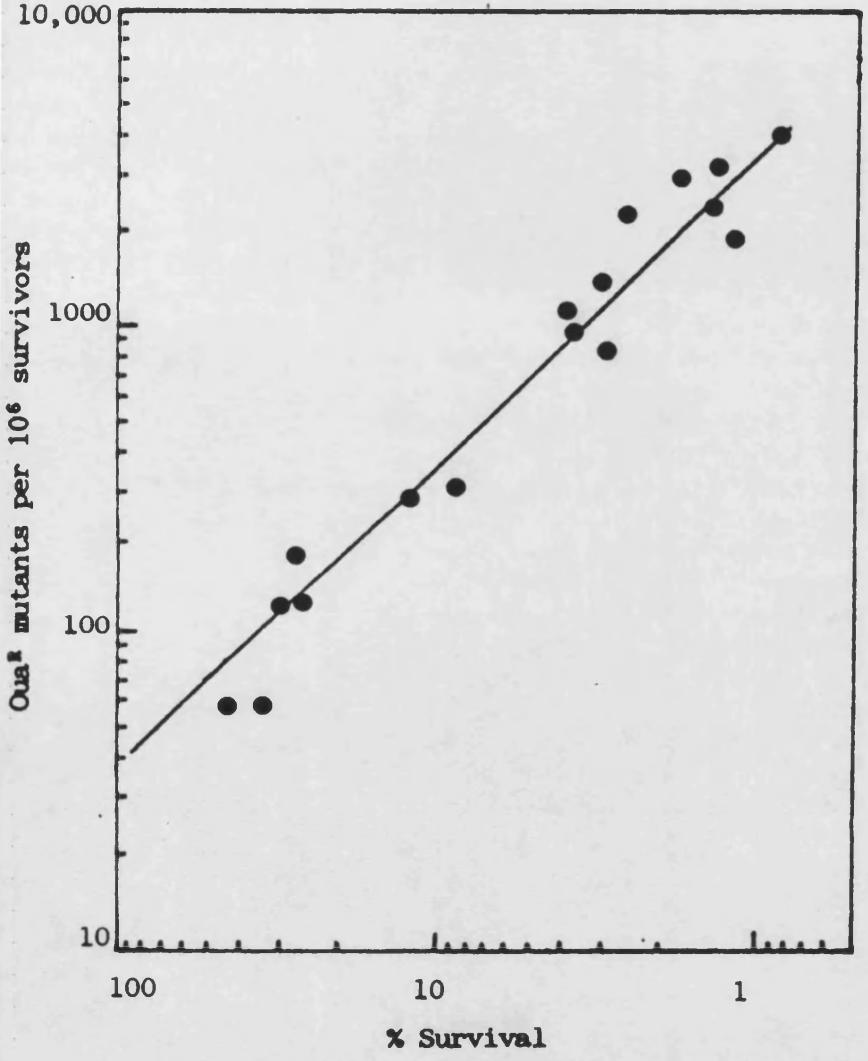


Figure 4.4. Reproducibility of the dose-response curve of the MNNG-induced mutation frequency to Oua^R of V79-379A cells grown in Hams F10 + 5% FCS (n=6 ± S.E.)



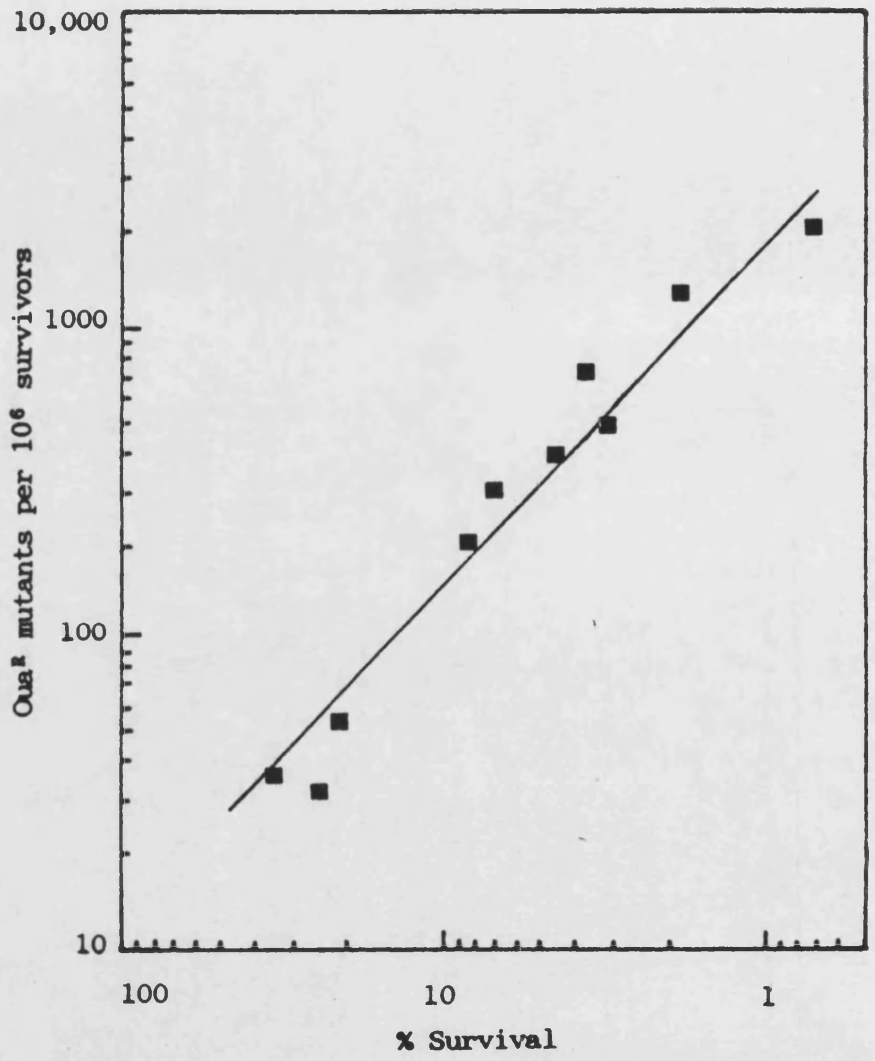


Figure 4.5. The relationship between MNNG-induced mutation frequency to Oua^R and percentage survival of CHO-K1 cells, (a), and V79-379A cells, (b), grown in Hams F10 + 5% FCS.

(1979) later used this method to compare the cytotoxic and mutagenic potencies of a number of different mutagens. Fig.4.5. shows this relationship to be linear for both CHO-K1 and V79-379A cells where the data used to present Figs.4.3. and 4.4. are plotted as cell survival against mutation frequencies, both on log scales.

4.2.3. The effect of adaptive pre-treatment on the MNNG induced mutation to Oua^R in CHO-K1 and V79-379A cells.

Adaptive pre-treatment of both cell lines was administered as previously described in 3.4.2. and as shown diagrammatically in Fig.4.14. At time zero (t=0) selection and survival plates were set up and the protocol described in 4.2.1. followed through. Care was taken to keep the control and pre-treated cells separated to avoid cross-contamination.

The effect of adaptive pre-treatment on the MNNG-induced mutation to Oua^R is shown in Figs.4.6. and 4.7. for CHO-K1 and V79-379A cells respectively. Each data point is comprised of three experiments and is expressed as mean mutation frequency ± standard error. Control values are also plotted for comparative purposes. Pre-treatment has reduced the MNNG-induced mutation to Oua^R when compared to control at each mutagen dose level for both cell lines. However, background mutation frequencies in pre-treated cell is much higher than in control cells i.e. $30x10^{-6}$ vs $0.91x10^{-6}$ for CHO-K1 cells and $30x10^{-6}$ vs $0.97x10^{-6}$ for V79-379A cells.

The data plotted in Figs.4.6. and 4.7. for pre-treated cells shows the M.F. with this background mutation subtracted thus showing only the 'challenge-induced' mutation to Oua^R rather than

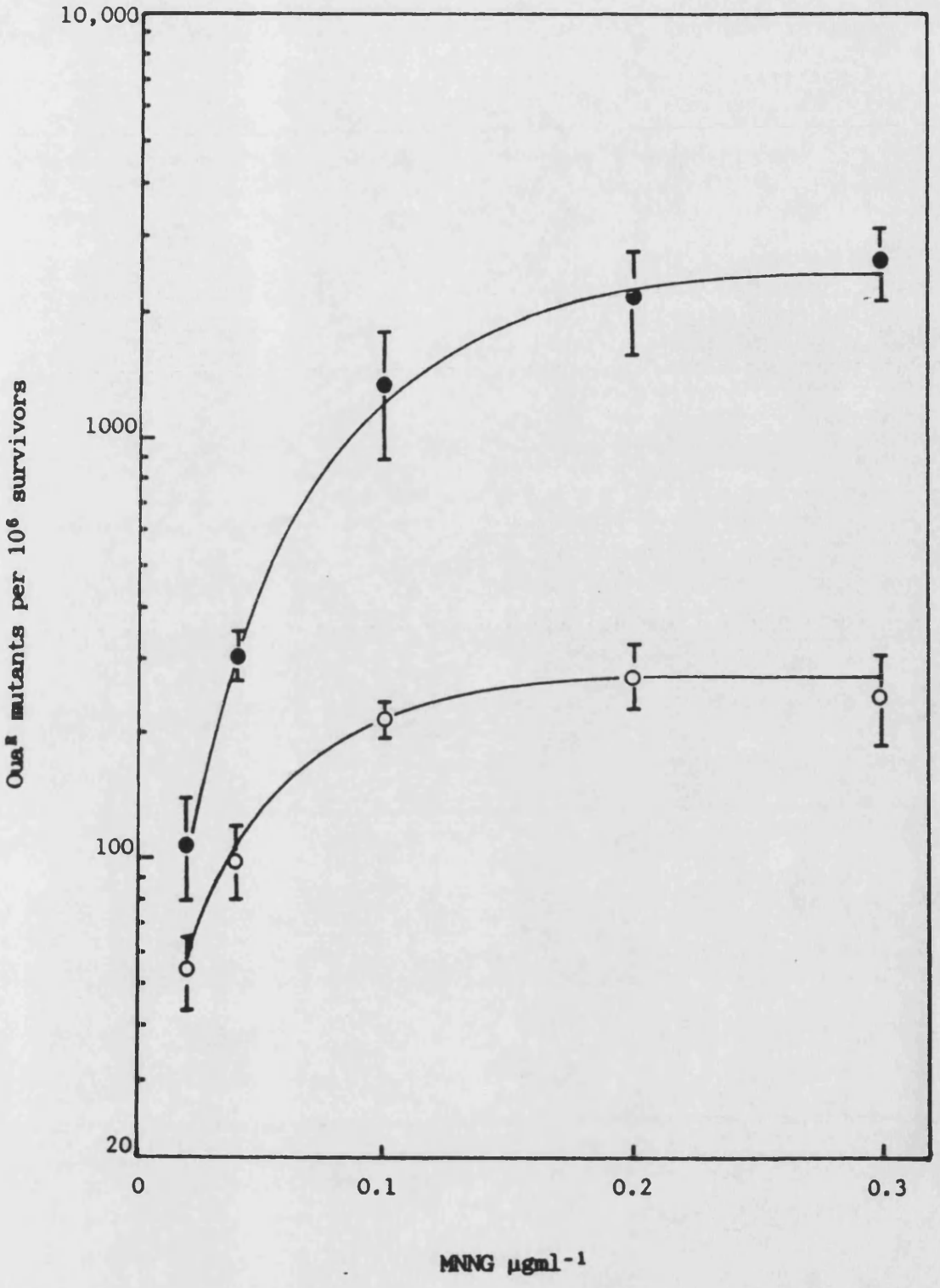


Figure 4.6. The effect of adaptive pre-treatment, by Protocol 2, on the dose-response curve of MNNG-induced mutation frequency to Oua^R, of CHO-K1 cells grown in Hams F10 + 5% FCS. Open and closed symbols represent pre-treated cells and control cells respectively. (n=3 ± S.E.)

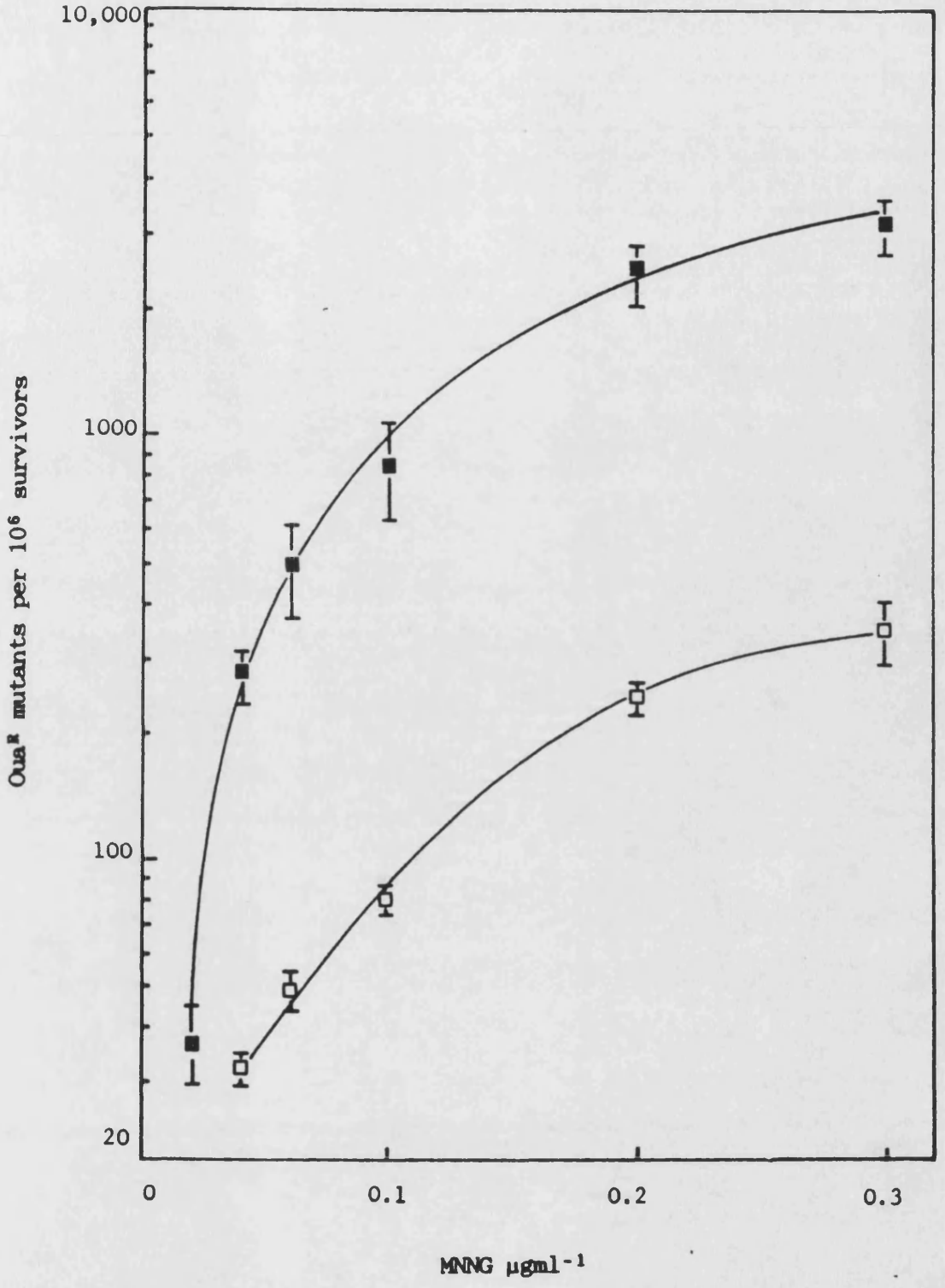


Figure 4.7. The effect of adaptive pre-treatment, by Protocol 2, on the dose-response curve of MNNG-induced mutation frequency to Oua^R, in V79-379A cells grown in Hams F10 + 5% FCS. Open and closed symbols represent pre-treated cells and control cells respectively. (n=3 ± S.E.)

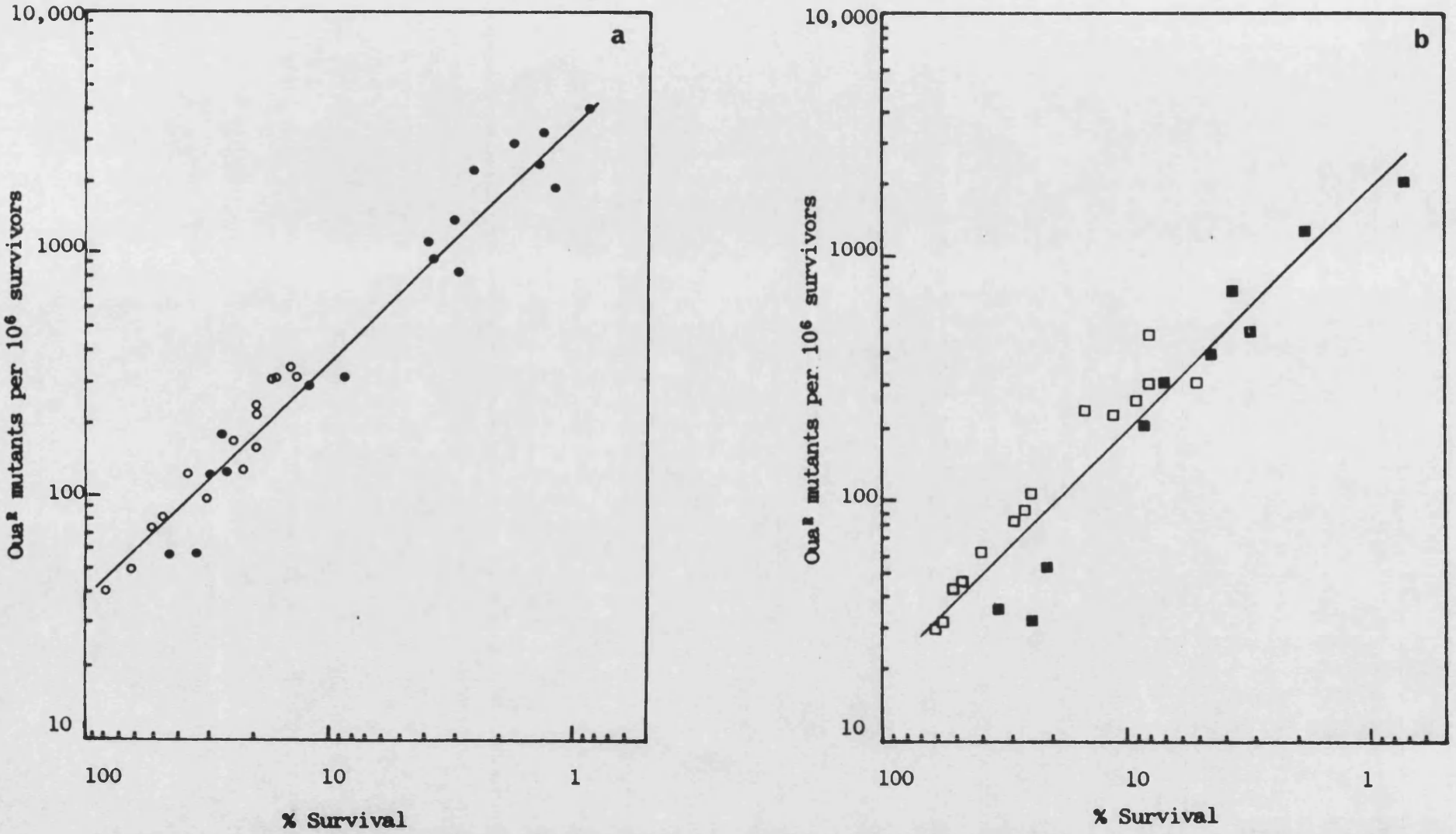


Figure 4.8. The effect of adaptive pre-treatment, by Protocol 2, on the relationship between MNNG-induced mutation frequency to Oua^R and percentage survival of CHO-K1 cells, (a), and V79-379A cells, (b) grown in Hams F10 + 5% FCS. Open and closed symbols represent pre-treated cells and control cells respectively.

the 'pre-treatment plus challenge-induced' mutation to Oua^R. Elevation of control mutation frequencies was also observed by Schwartz and Samson, (1983) when they pre-treated CHO cells with MNNG and then determined the induced mutation to 6-thioguanine resistance. If the subtraction of background is not performed, however, there still remains a substantial decrease in the mutation frequencies of pre-treated cells over control e.g. for a challenge dose of 0.2 μgml^{-1} values are 3073 vs 380 (8-fold) and 2014 vs 260 (7.7-fold) for CHO-K1 and V79-379A cells respectively (Figs.4.6. and 4.7.). Therefore from these data it is apparent that after pre-treatment with MNNG both CHO-K1 and V79-379A cells yield lower mutation frequencies to Oua^R, on a dose basis, compared to those of un-treated control cells (Figs.4.3. and 4.4.). However analysis of the mutation data at equicytotoxic doses, (Durrant *et al.*, 1981; Friedman and Huberman, 1980; Ishida and Takahashi, 1987), revealed these cells to be as equally mutable as their respective control cells (Figure 4.8.).

4.3. The effect of adaptive pre-treatment on the growth parameters of CHO-K1 and V79-379A cells.

One of the most favourable characteristics of Chinese hamster cell lines, making them very useful to these studies, is their ability to grow in a consistent and reproducible fashion. It was therefore important to establish the growth parameters of both CHO-K1 and V79-379A cell lines and the effect adaptive pre-treatment had on these parameters.

4.3.1. Determination of the growth parameters of CHO-K1 and V79-379A cells.

Inocula of $2x10^5$ cells were sub-cultured into a number of $25cm^2$ T/C flasks each containing 5ml Hams F10 + 5% FCS, gassed with 5% CO_2 in air and incubated at 37.5°C. At intervals a flask was removed from the incubator and the total cell number determined. The medium was withdrawn and placed in a 10ml graduated round-bottomed centrifuge tube (MSE Scientific Instruments). The cell monolayer was then rinsed with two 1ml portions of trypsin solution (2.3.3.), adding each rinse in turn to the centrifuge tube. The cells were detached from the flask by incubating with a further 1ml trypsin solution. After the appropriate incubation time (2.6.2.) the flasks were removed from the incubator, gently tapped on the heel of the hand to dislodge the cells and the resultant cell suspension transfered to the centrifuge tube. The flask was then rinsed with two 1ml portions of PBS to remove the residual cells. Complete removal was confirmed by examination of the flasks using phase-contrast microscopy. Cell losses due to adherance were minimised by the use of a single siliconised Pasteur pipette for all the above transferances (2.5.4.). Cells were concentrated by centrifugtion (1500 rpm for 5 min) and resuspended in 0.5ml of the supernatant. The suspension was adjusted to an expected density of $2.5x10^5$-$1.5x10^6$ cells ml^{-1} with PBS and the final volume noted. The cell density was determined by haemocytometer count and the total cell number calculated.

Both CHO-K1 and V79-379A cell lines exhibit 'classical' growth kinetics, growth being conventionally divided into three distinct phases:

i) Lag phase : this is the time following sub-culture and

seeding where there is little evidence of an increase in cell number. It is a period during which the cell replaces elements of the glycocalyx lost during trypsinisation, attaches to the substrate, and spreads out (Freshney, 1983).

ii) Log phase : this is the period where cell numbers increase exponentially. It follows the lag phase and is also known as the exponential growth phase.

iii) Stationary phase : towards the end of the log phase the culture becomes confluent i.e. all the available growth surface is occupied and the cells are in contact with neighbouring cells. Hence a reduction in cell growth rate follows (Freshney, 1983).

Growth curves are constructed by plotting the total number of cells in each flask, on a log scale, against the time elapsed from seeding, having incubated the cells at 37.5°C in a 5% CO_2 in air atmosphere. Figures 4.9. and 4.10. show typical growth curves of CHO-K1 and V79-379A cells respectively. From such curves two useful parameters can be obtained namely the lag time, the time taken for growth to commence, and the population doubling time (T). The latter is the time taken for the culture to increase its numbers two-fold during the log or exponential growth phase and is the inverse of k, the exponential growth rate constant. This should not be confused with the generation or cell cycle time

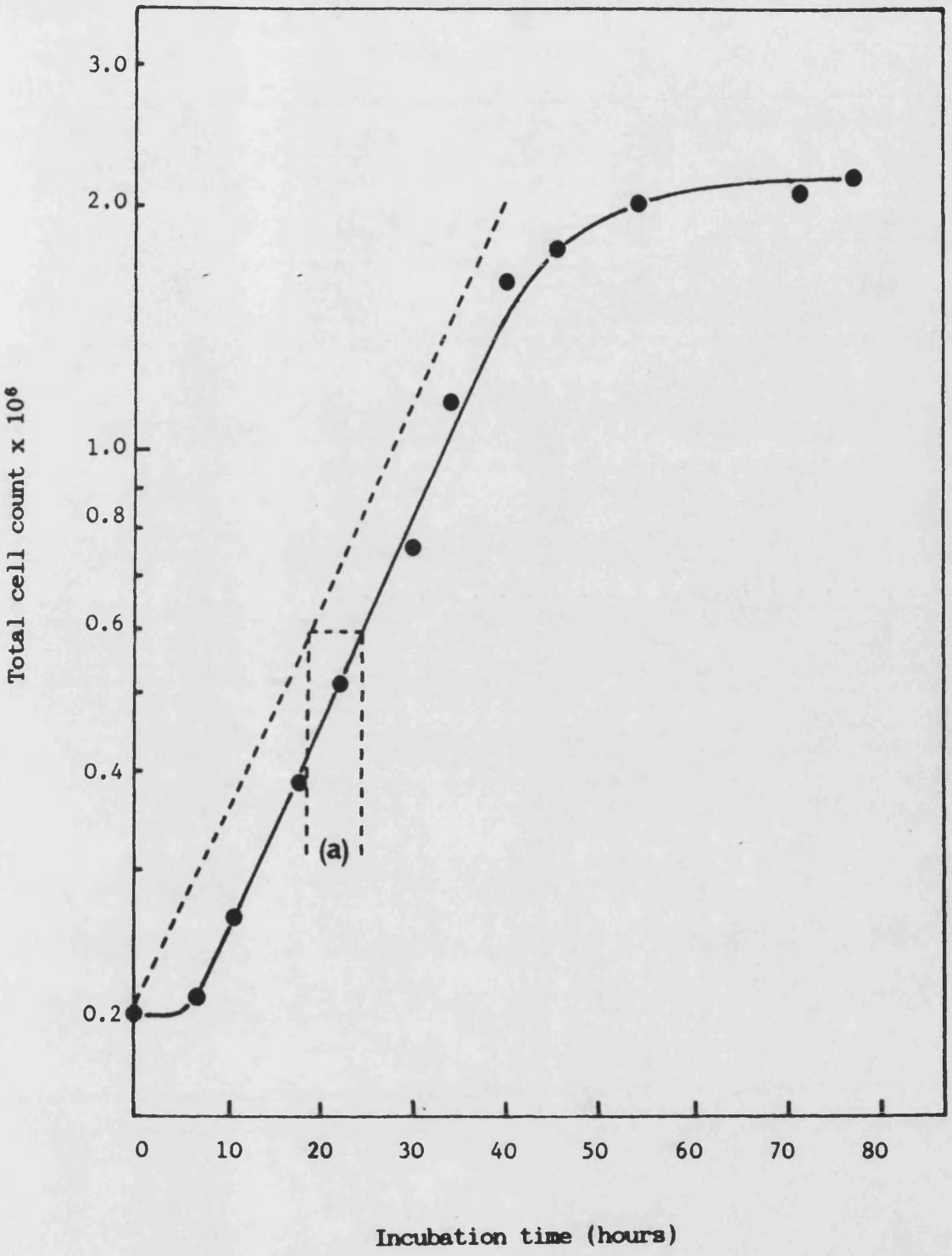


Figure 4.9. Growth curve of CHO-K1 cells grown in Hams F10 + 5% FCS.

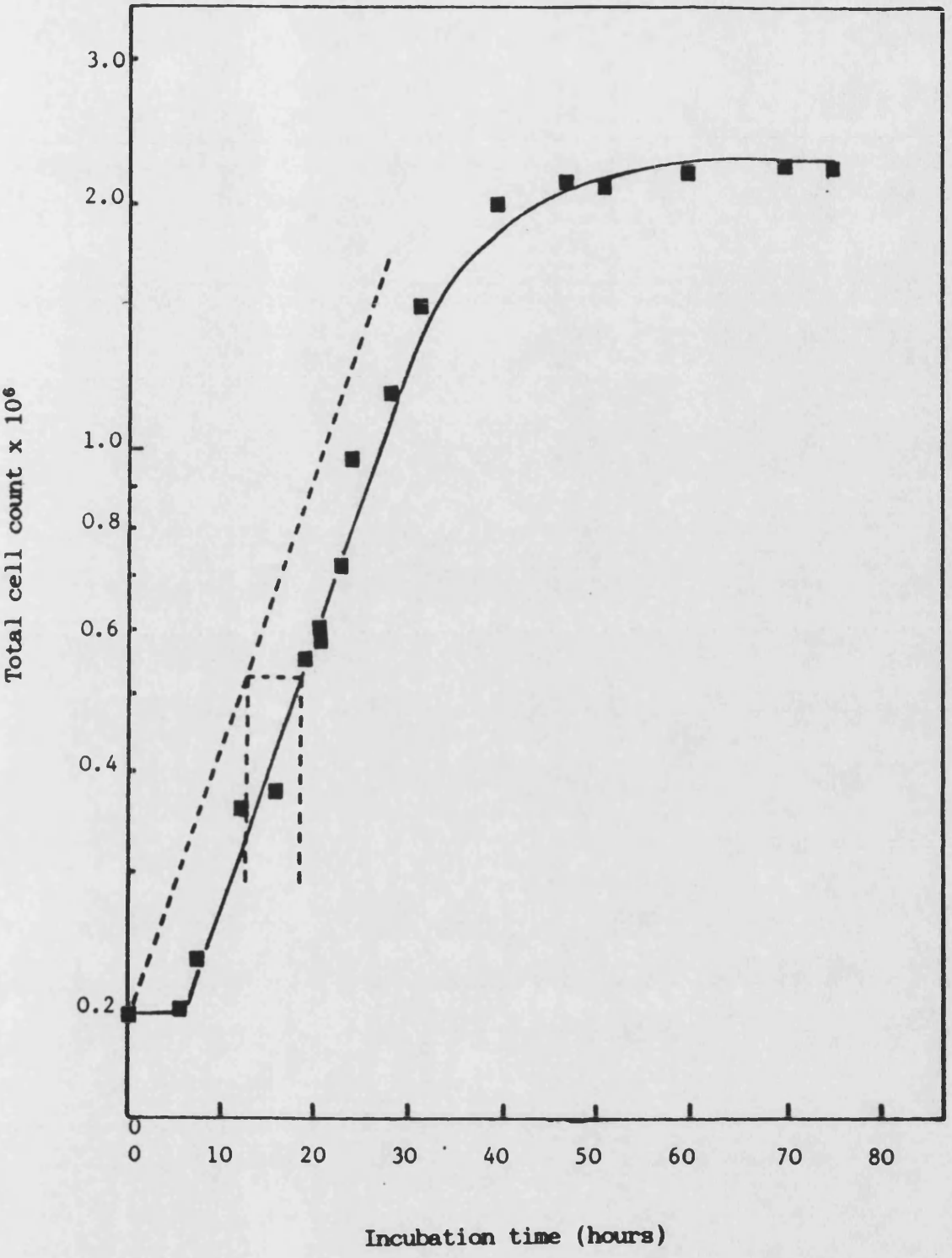


Figure 4.10. Growth curve of V79-379A cells grown in Hams F10 + 5% FCS.

Table 4.2. Growth parameters of CHO-K1 and V79-379A cells, grown in Hams F10 + 5% FCS.

Cell line	Lag time (hrs)	k value (hr^{-1})	T (hrs)	r
CHO-K1	6	0.069	14.4	0.99
V79-379A	6	0.088	11.4	0.98

k = Exponential growth rate constant.
T = Population doubling time.
r = Correlation coefficient.

which is established by measuring the transit of a population of cells through the cycle until they return to the same point in that cycle. During the exponential or log phase of growth, an initial population of N_o cells will, after a time t minutes, be increased to N_t cells according to the following equation

$$N_t = N_o\ 2^{kt}$$

or in its logarithmic form: $\text{Log}N_t = \text{Log}N_o + k.t.\text{Log}2$

where:

N_o = cell number at time 0 N_t = cell number at time t

k = exponential growth rate constant.

The growth parameters for both CHO-K1 and V79-379A cells are shown on Table 4.2. along with the correlation and regression values calculated from the log phase of each cell line. The values of slope and r (correlation coefficient) were calculated using a BBC Master microcomputer running the INSTAT statistical package (Reading University), which included a correlation and regression program. The values of T for both lines, i.e. 14.4 hours for CHO-K1 and 12.3 hours for V79-379A cells (Table 4.3.), are in good agreement with the literature values of 10 to 16 hours reported for various CHO and V79 cell lines. (Bradley *et al.*, 1981; Hsie *et al.*, 1981; Kao and Puck, 1974.).

4.3.2. <u>The effect of adaptive pre-treatment on the growth parameters of CHO-K1 and V79-379A cells.</u>

To determine the effect of adaptive pre-treatment on the growth parameters of these cell lines, cultures were set up and pre-treated with repeated doses of mutagen as detailed in 3.4.2. and Fig.4.14. At t=0 the cells were dispersed by trypsinisation and the cell density determined. $2x10^5$ cells were inoculated into a

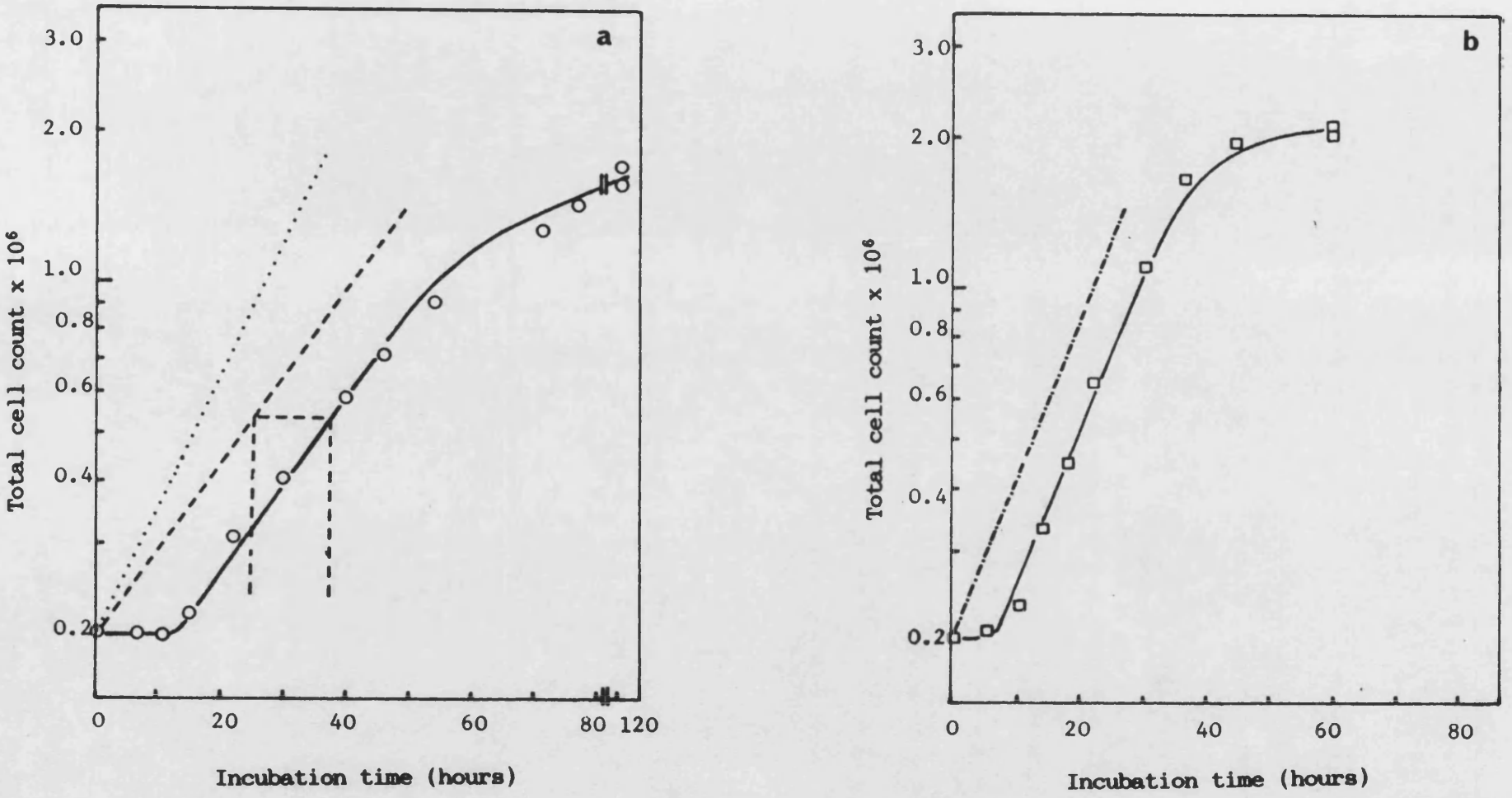


Figure 4.11. The effect of adaptive pre-treatment, by Protocol 2, on the growth curves of CHO-K1 cells, (a), and V79-379A cells, (b). A regression line for untreated control cells (.....) is included to illustrate the relative growth rates of pre-treated cells (-----) and control cells.

Table 4.3. The effect of adaptive pre-treatment, by Protocol 2, on the growth parameters of CHO-K1 and V79-379A cells, grown in Hams F10 + 5% FCS.

Cell line	Lag time (hrs)	k value (hr^{-1})	T (hrs)	r
CHO-K1	6	0.069	14.4	0.99
CHO-K1 + adaptive pre-treatment	14	0.043	23.3	0.99
V79-379A	6	0.088	11.4	0.98
V79-379A + adaptive pre-treatment	6	0.081	12.3	0.99

k = Exponential growth rate constant.
T = Population doubling time.
r = Correlation coefficient.

number of flasks and growth curves for each cell line were constructed as described in 4.3.1.

The effects of adaptive pre-treatment are shown in Fig.4.11. and also on Table 4.3. Pre-treatment has increased the doubling time of CHO-K1 cells by 8.9 hours from 14.4 hours to 23.3 hours, whereas the doubling time of V79-379A cells was increased only slightly from 11.4 to 12.3 hours. Fig 4.11.a, illustrates the effect of pre-treatment on CHO-K1 cells by comparing regression lines for control and pre-treated cells. Tobey an Crissman, (1975) showed CHO cells to ave an increased population doubling time following mutagen treatment whilst Roberts and Ward, (1973) reported an increase in theis parameter in MNU-treated cultures of V79 cells.

The lag time of CHO-K1 cells was increased by 8 hours over the control value of 6 hours following adaptive pre-treatment, whereas that of V79-379A cells was identical to the lag time of untreated control cells.

A summary of the pre-treatment protocol and the determination of growth, cytotoxicity and mutation in both CHO-K1 and V79-379A is shown in Fig.4.14.

4.4. The effect of cell synchrony on the MNNG-induced cytotoxicity and mutation to Oua^R of CHO-K1 cells.

Barranco and Humphrey, (1971) reported that the sensitivity of CHO cells to MNNG fluctuates through the cell cycle. It would seem likely, therefore, that a treatment causing a change in cell cycle distribution may generate an apparent change in the MNNG sensitivity of the whole population. MNNG has been shown to delay the progression of the cell cycle of Chinese

hamster lines (Barranco and Humphrey, 1971; Roberts and Ward, 1973) which is ultimately seen as an increase in the lag time of the growth curve. It was decided, therefore, to determine the MNNG-induced cytotoxicity and mutation to Oua^R for a population of synchronised CHO-K1 cells to establish whether the adaptive pre-treatment protocol used had induced synchrony within the population. The results were compared to the responses of asynchronous cultures (3.2.1. and 4.2.1.) and to those of pre-treated asynchronous cultures (3.4.2. and 4.2.3.). Since adaptive pre-treatment affected the lag time of CHO-K1 cells, but not V79-379A cells, only the former were used in these experiments.

4.4.1. Synchrony of CHO-K1 cells.

Synchrony was achieved using the protocol of Zwanenburg, (1983), who made use of the fact that CHO cells round up and become less firmly attached to the growing surface when they enter mitosis. The mitotic cells can be shaken off the monolayer, collected and concentrated by centrifugation. The advantages of this 'shake-off' method over other techniques is that there is no need for the addition of chemicals to enrich the initially randomly-growing population, with cells in a specific stage of the cell cycle e.g. the addition of an excess of thymidine to obtain cells at the beginning of S-phase (Barranco and Humphrey, 1971).

Two days prior to the experiment four CHO-K1 cultures were initiated by inoculating $5x10^5$ cells into 150ml culture bottles each containing 15ml Hams F10 + 5% FCS. After 48 hours growth at 37.5°C, in a 5% CO_2 in air atmosphere, the medium was removed from the cultures and replaced with 10ml of fresh, pre-warmed Hams F10

+ 5% FCS. The bottles were then shaken gently to remove dead cells and loosely attached interphase cells. The medium was removed and again replaced with 10ml fresh media. After 30 minutes the first 'shake-off' was performed by gentle circular agitation, the cells being collected by centrifugation of the removed media at 1000rpm for 5 min. A further 10ml medium were added to each culture bottle and re-incubated for 1 hour. The cells collected were kept on ice, in medium lacking FCS, until needed. This prevented the cells from progressing through the cell cycle. This procedure was repeated twice again i.e. a total of three 'shake-offs', the cells were pooled and used immediately following the last collection.

4.4.2. The effect of synchrony on the MNNG-induced cytotoxicity and mutation to Oua^R in CHO-K1 cells.

Two days prior to the experiment, cultures of CHO-K1 cells were set up in four 150ml culture bottles each containing 15ml Hams F10 + 5% FCS, gassed with CO_2 in air and incubated at 37.5°C. After 48 hours growth, cells were synchronised and collected by the method of Zwanenburg, (1983) (4.4.1.). Following cell density determination of each population, dilutions were carried out as required, to set up selection and survival plates. After 7 days incubation at 37°C in a 5% CO_2 in air atmosphere, the plates were stained, randomised and scored 'blind'. The results of both survival and mutation to Oua^R for pre-treated and control synchronous cells are shown in Fig.4.12. Also included in this Figure, for comparative purposes, are the dose-response curves of unsynchronised control cells and pre-treated unsynchronised control cells, as previously plotted in Figs.3.7., 4.3. and 4.6.

Fig.4.12.a. shows survival profiles similar to those seen for

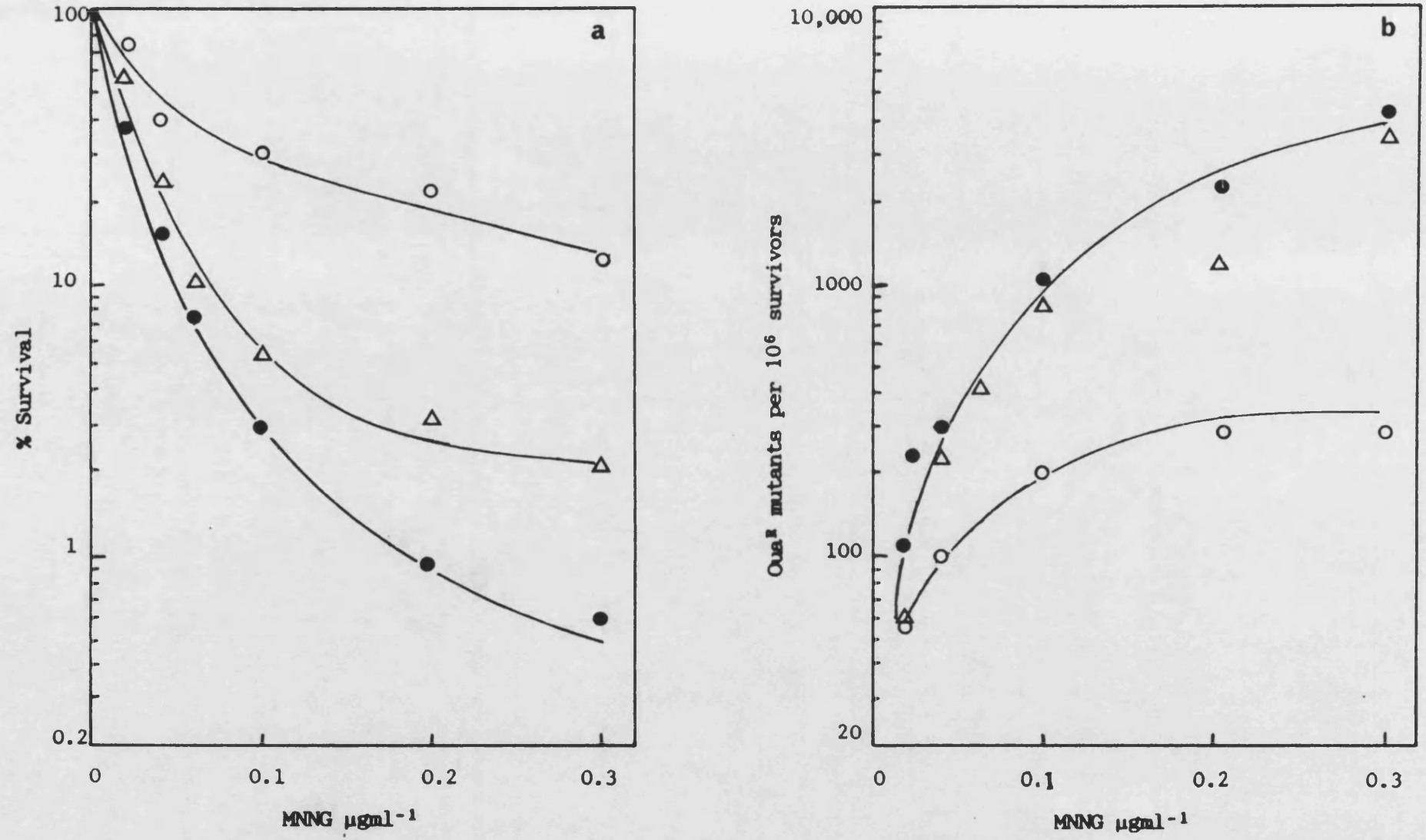


Figure 4.12. The effect of cell synchrony on the MNNG dose-response curves of survival and induced mutation frequency to Oua^R, for CHO-K1 cells grown in Hams F10 + 5% FCS. (●) = control; (△) = synchronised cells; (○) = cells pre-treated by Protocol 2.

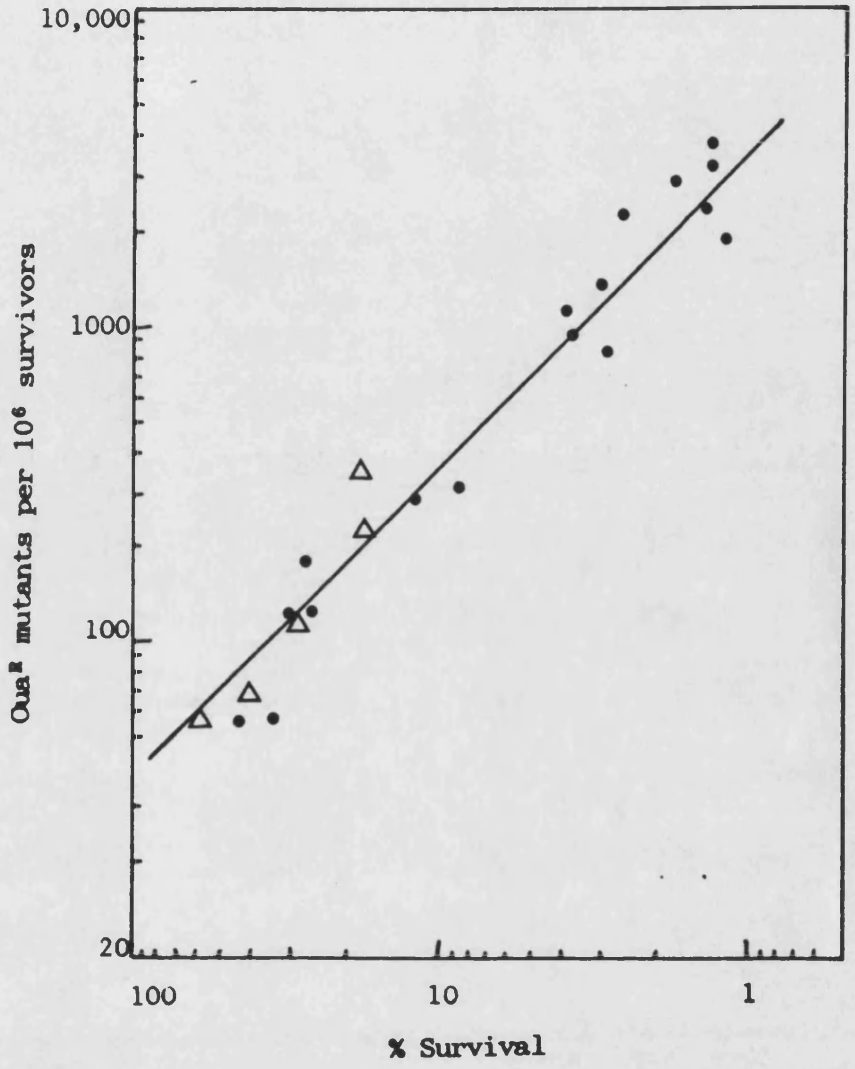
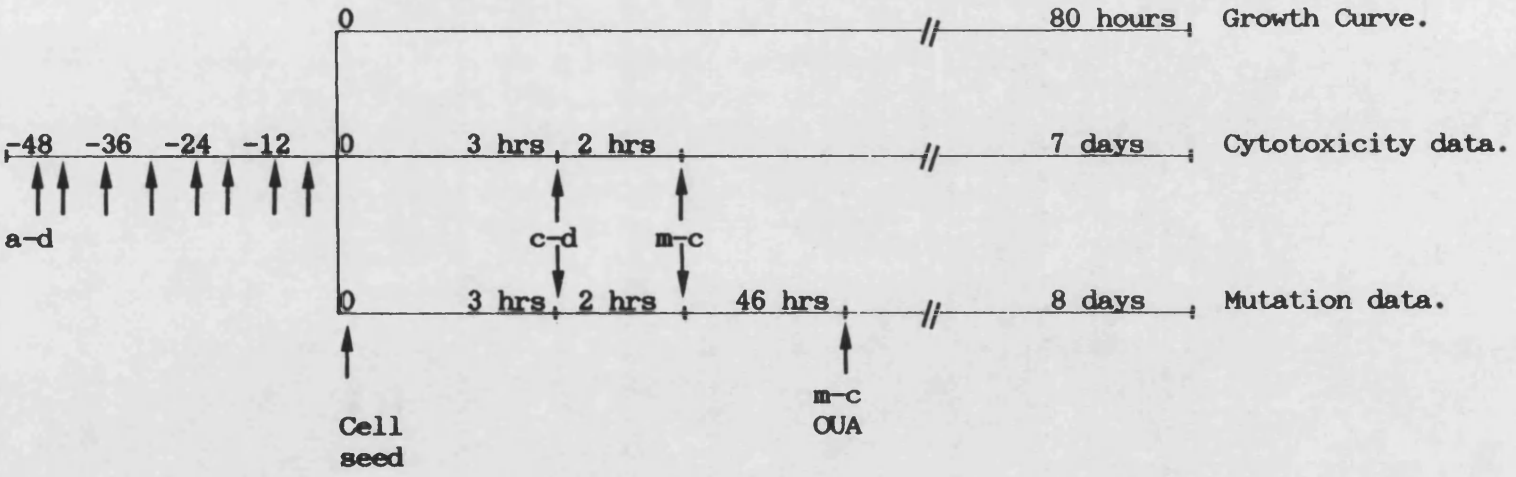


Figure 4.13. The effect of cell synchrony on the Relationship between induced mutation frequency to Oua^R and percentage survival, of CHO-K1 cells grown in Hams F10 + 5% FCS. Open and closed symbols represent synchronised cells and control cells respectively.

Figure 4.14. Summary of adaptation pre-treatment Protocol 2.



a-d : MNNG adaptive dose (0.01 $\mu g ml^{-1}$).
c-d : MNNG challenge doses (0.02-0.3 $\mu g ml^{-1}$).
m-c : Medium change
OUA : Mutant selection medium - Hams F10 + 5% FCS + 1mM ouabain.

asynchronous cells (Fig.3.7.) but different to those seen for pre-treated unsynchronised cells. From this Figure a D_{37} value of 0.028 $\mu g ml^{-1}$ MNNG for synchronised CHO-K1 cells is derived. This value is 1.33-fold greater than that of unsynchronised cells i.e. 0.021 $\mu g ml^{-1}$ MNNG, but is 1.5-fold less than the D_{37} value of 0.042 $\mu g ml^{-1}$, derived for adaptively pre-treated CHO-K1 cells

Synchronised CHO-K1 cells exhibit similar mutation frequencies to unsynchronised cells, both on a dose basis and on an equicytotoxic basis (Figs.4.12.b. and 4.13.).

4.5. Conclusions.

From the results reported in this chapter the following can be concluded.

1) MNNG, in the range 0.02-0.3 $\mu g ml^{-1}$ induced mutation at the Na^+/K^+ATPase locus in a reproducible and dose-dependent fashion for both CHO-K1 and V79-379A cell lines (Figs.4.2., 4.3. and 4.4.).
2) The spontaneous (vehicle control) mutation frequencies were low and in the order of $1x10^{-6}$.
3) Adaptive pre-treatment resulted in a reduced MNNG-induced mutation to Oua^R, on a dose basis, for both CHO-K1 and V79-379A cell lines (Figs.4.6. and 4.7.). However, when the data was analysed at equicytotoxic doses the pre-treated cells were found to be as equally mutable as untreated cells (Fig.4.8.).
4) The spontaneous (vehicle control) mutation frequencies of pre-treated cells were higher than control cells in the order of $3x10^{-5}$.
5) The growth of CHO-K1 and V79-379A cells can be defined according to two parameters i.e. lag time and population doubling time. For CHO-K1 the values are 6 hours and 14.4 hours respectively, and for V79-379A cells are 6 hours and 11.4 hours (Table 4.2).
6) Adaptive pre-treatment of V79-379A cells did not alter the lag time but slightly increased the population doubling time from 11.4 hours to 12.3 hours (Table 4.3.).
7) Adaptive pre-treatment of CHO-K1 cells increased the lag time by 8 hours, from 6 to 14 hours, and also increased the population doubling time by 8.9 hours from 14.4 hours to 23.3 hours

(Table 4.3.).

8) Synchronised CHO-K1 cells exhibit a D_{37} value of 0.028 μgml^{-1} MNNG, which is 1.33-fold greater than the D_{37} value for unsynchronised cells (0.021 μgml^{-1}) and 1.5-fold less than the D_{37} value for unsynchronised pre-treated cells (0.042 μgml^{-1}). the induced mutation to Oua^R was not significantly altered by synchrony.

These conclusions are discussed in Chapter 8.

CHAPTER 5. THE ISOLATION AND CHARACTERISATION OF CHO-K1 AND V79-379A CLONES SENSITIVE OR RESISTANT TO THE CYTOTOXIC EFFECTS OF MNNG.

5.1. Introduction.

CHO-K1 and V79-379A cells exposed to MNNG exhibit a dose-dependent cell killing which is seen to be bi-phasic (3.2.1. and 3.2.2.). This could indicate the presence of at least two cell sub-populations each with different sensitivites to MNNG. It is suggested, therefore, that MNNG pre-treatment of both cell lines may enrich the overall population with the more resistant sub-population. This will result in an enhanced survival (reduced kill) to subsequent mutagen challenge, a theory also proposed by Goth-Goldstein, (1987), in a recent publication. A number of authors, using various cell lines, have isolated clones that have survived high doses of mutagen and which are subsequently more resistant to mutagen challenge (Baker *et al.*, 1979; Friedman and Huberman, 1980; Goth-Goldstein, 1987a; Goth-Goldstein and Hughes, 1987a; Goldmacher *et al.*, 1986; Ishida and Takahashi, 1987). These clones are probably representative of the mutagen resistant sub-population of the chosen cell line.

It was proposed, therefore, to isolate the two sub-populations evident from the dose-response curves of both CHO-K1 and V79-379A cells (Fig.3.2. and 3.3.), determine the growth parameters, MNNG-induced cytotoxicity and mutation to Oua^R and compare the results to the values obtained from their respective parental lines. The effects of pre-treatment on these variants was determined and compared to those observed with the parental control and pre-treated cells.

5.2. Materials.

The isolation of sensitive and resistant sub-populations required a number of special materials not listed in Chapter 2:

Glass selection cylinder: (5mm internal diameter x 8mm external diameter x 10mm high) with flame-rounded edges, prepared from a length of Pyrex glass tubing. Sterilised by dry heat at 160°C for 1 hour.

Silicone grease: (Edwards High Vacuum Ltd., Crawley Sussex.), dry heat sterilised at 160°C for 1 hour.

Polyester mesh: 17μm Polymer mesh (PES 17/6. Plastok Associates Ltd, Birkenhead). Mesh circles were cut using an 85mm template. Notches were made on the edge of each disc to facilitate matching of replicas.

Glass balls: 4.5 to 5.5mm diameter (BDH chemicals Ltd., Poole, Dorset).

Preparation of materials for replica plating:

Systematic preparation of polyester discs and glass balls was essential for consistent results. Following initial use, both polyester discs and glass balls may be re-cycled and used repeatedly;

Initial preparation of glass balls: the glass balls were subjected to an overnight soak in 10% v/v nitric acid was followed by a thorough wash in running water (2 hours). A 2 hour soak in sodium hypochlorite solution (12% available chlorine), diluted 1/50 in water, was followed by a thorough wash in running water. The glass balls were then boiled in detergent (2% RBS 25. Chemical concentrates Ltd., London) for 5 minutes. The glass balls were washed for several hours in running water and then rinsed with

four changes of distilled water and drained well. They were placed in a beaker, covered with foil and sterilised for 1 hour at 160°C.

Re-cycling of glass balls: after removal from the culture medium the glass balls were soaked for two hours in detergent, washed for two hours in running water then rinsed with four changes of distilled water. After dry heat sterilisation the balls were ready for re-use.

Polyester: initial preparation and re-cycling. A batch of polyester discs was soaked in dilute bleach for 30 minutes, washed in running water for two hours and boiled in detergent for 5 minutes. The discs were washed overnight in running water, rinsed with four changes of distilled water and drained well. Sterilisation was performed by placing each disc between a filter-paper 'sandwich' within a well fitting autoclave bag. It was important to keep the mesh discs flat throughout the sterilisation procedure to maintain their structural integrity. The sealed bags were then placed within an autoclave and sterilised at 121°C for 15 minutes.

5.3. Cell isolation procedures.

5.3.1. Isolation of MNNG resistant clones CHO-K1R and V79-379AR by ring cloning.

Isolation of MNNG resistant clones of both cell lines was carried out by following the ring cloning technique of Kao and Puck, (1974).

48 hour cultures of either CHO-K1 or V79-379A were trypsinised and the cell densities of each suspension determined. Suspensions were diluted to a density of $2x10^4$ cells ml^{-1} and 0.1 ml aliquots of this suspension were transfered to a number of 90mm

T/C petri dishes each containing 10ml of Hams F10 + 5% FCS. Following three hours incubation, at 37.5°C in a 5% CO_2 in air atmosphere, 0.3 μgml^{-1} MNNG was added and the plates re-incubated for two hours. After this time the medium was replaced with fresh, pre-warmed Hams F10 + 5% FCS and the plates re-incubated for seven days. At this dose level there should be an approximate survival rate of 1% i.e. about 20 colonies per plate (Figs.3.2. and 3.3.). The location of each colony was marked with a glass marker on the underside of the plate. Each colony was examined under an inverted microscope (2.1.) and a colony which was sufficiently isolated from other cells was chosen for clone isolation for each cell line. The medium in the plates was removed and a glass selection cylinder placed directly over the centre of each colony. The cylinder was caused to adhere to the bottom surface of the plate by application of a thin layer of silicone grease to the bottom edge and then pressed firmly to the plate, making a seal with the plastic. Both the cylinder and grease had been previously sterilised by dry heat (5.2.). The cylinder was rinsed with a few drops of trypsin solution and the wash discarded. Cells were dispersed by adding a few drops of trypsin and incubating for 5 minutes at 37.5°C. the cells were suspended by gentle aspiration with a Pasteur pipette and transfered to 25cm^2 T/C flasks containing 5ml Hams F10 + 5% FCS . MNNG at 0.3 μgml^{-1} was added and the cells allowed to grow to 75% confluence i.e. about 7-9 days. After this time the cells were dispersed and the cell density determined. The cells were once more plated out but this time only 500 per plate were seeded since the cells were assumed to be more resistant to the lethal effects of MNNG. After 3 hours incubation 0.3 μgml^{-1} MNNG was added to each plate and the cells allowed to grow for 7-9

days. Resistant colonies of each cell line were once again isolated and allowed to grow in 25cm^2 T/C flasks each containing 5ml Hams F10 + 5% FCS plus 0.3 μgml^{-1} MNNG. After 7-9 days growth the cells were dispersed, counted and sub-cultured into medium lacking MNNG. Once 75% confluence was reached the cells were prepared for strorage over liquid nitrogen (2.6.5.). The double cloning procedure for resistant mutants was recommended by Thompson, (1978). The isolated MNNG-resistant clones from each cell line were designated as CHO-K1R and V79-379AR (R = survivors of a 0.3 μgml^{-1} MNNG dose).

5.3.2. Isolation of MNNG-sensitive clones CHO-K1S and V79-379AS by replica plating on polyester mesh discs.

Isolation of MNNG-resistant clones is carried out by removing surviving colonies from a culture subjected to a specific mutagen dose. Isolation of MNNG-sensitive clones is, however, a more difficult process. Since the clones sought are specifically those with an increased sensitivity to MNNG the only technique available for distinguishing these cells is one that kills them. One solution to this problem is to use the technique of replica plating. Commonly used with bacteria and yeasts this technique has been less easily applied to mammalian cells. Replica plating involves the inoculation of low densities of cells into petri-dishes, allowing single cell growth, the resultant colonies being copied onto a suitable material. After mutagen treatment, the most sensitive colonies can be identified and isolated from the corresponding position on the untreated replica. Esko and Raetz, (1978), initially used filter paper discs for replica plating of CHO cells but found this technique unsuitable for other

cell lines. In a later publication Raetz *et al.*, (1982) reported a more efficient method of replica plating using polyester mesh discs to produce high quality replicas of CHO cells and even relatively non-adherent myeloma and hybridoma cell lines.

The method of Raetz *et al.*, (1982) was therefore used to isolate MNNG-sensitive clones from the CHO-K1 and V79-379A cell lines.

The procedure for both cell lines was identical. Two days prior to the experiment cultures were set up by inoculating $5x10^5$ cells into 150ml culture bottles each containing 15ml of Hams F10 + 5% FCS, gassed with CO_2 in air and then incubated at 37.5°C. After 46-48 hours growth the cells were dispersed with trypsin, the cell density determined and the suspensions serially diluted to a density of $1x10^3$ cells ml^{-1}. 0.1ml aliquots of these suspensions were transfered into a number of 90mm T/C petri dishes each containing 10ml of Hams F10 + 5% FCS. The cells were evenly distributed by circular agitation and then incubated for 3 days. After this time four 85mm polyester discs were overlayed onto the partially developed colonies. The disc notches were aligned prior to overlaying and the position marked on the underside of each petri dish. The discs were weighed down with a single layer of sterile glass balls to ensure close contact between colonies and discs. CHO-K1 and V79-379A grow normally in monolayers, but given a physical matrix to adhere to, will grow vertically (Raetz *et al.*, 1982). After 3 days growth the medium and glass balls were tipped from the culture dishes and 5ml pre-warmed PBS(A) was added. The now floating discs were separated and transfered to 90mm T/C dishes containing 10ml of pre-warmed Hams F10 + 5% FCS. To two of the replica containing dishes 0.02 $\mu g ml^{-1}$ MNNG was added. Care was

taken not to use the top replica for the mutagen treatment since incomplete transfer of all colonies, to this disc, was sometimes observed. Plates were re-incubated for 3 days after which the treated replicas and one of the master copies were stained. The stained discs were compared and the position of absent colonies on the MNNG-treated discs scored on the control master disc. Once identified the corresponding live colony on the unstained master plate was located and isolated by ring cloning (5.3.1.). The isolated colonies were grown in 25cm^2 T/C flasks containing 5ml Hams F10 + 5% FCS, at 37.5°C in a 5% CO_2 in air atmosphere, until 75% confluence was reached, after which the cells were sub-cultured in preparation for later cell storage over liquid nitrogen (2.6.5.).

Plates 5.1. and 5.2. show the master, replica and mutagen treated replicas of CHO-K1 and V79-379A cells respecively. A few colonies have failed to transfer along the edges of some discs because the mesh was unevenly cut. The colony isolated from the unstained master disc (not shown) is indicated on the stained master discs. According to the dose-response curves shown in Figs 3.2. and 3.3. a dose level of 0.02 $\mu g ml^{-1}$ MNNG should result in an approximate 60% kill. This does not seem to hold true for the partially developed colonies found on each disc. Therefore only the colonies that had completely disappeared were selected. Replica 2 of Plate 5.1., however, shows the colonies to be less dense than the corresponding untreated replica possibly indicating a cytotoxic effect of MNNG but without complete killing of the colony.

The MNNG-sensitive clones derived from each cell line were designated CHO-K1S and V79-379AS (S = colonies most sensitive to

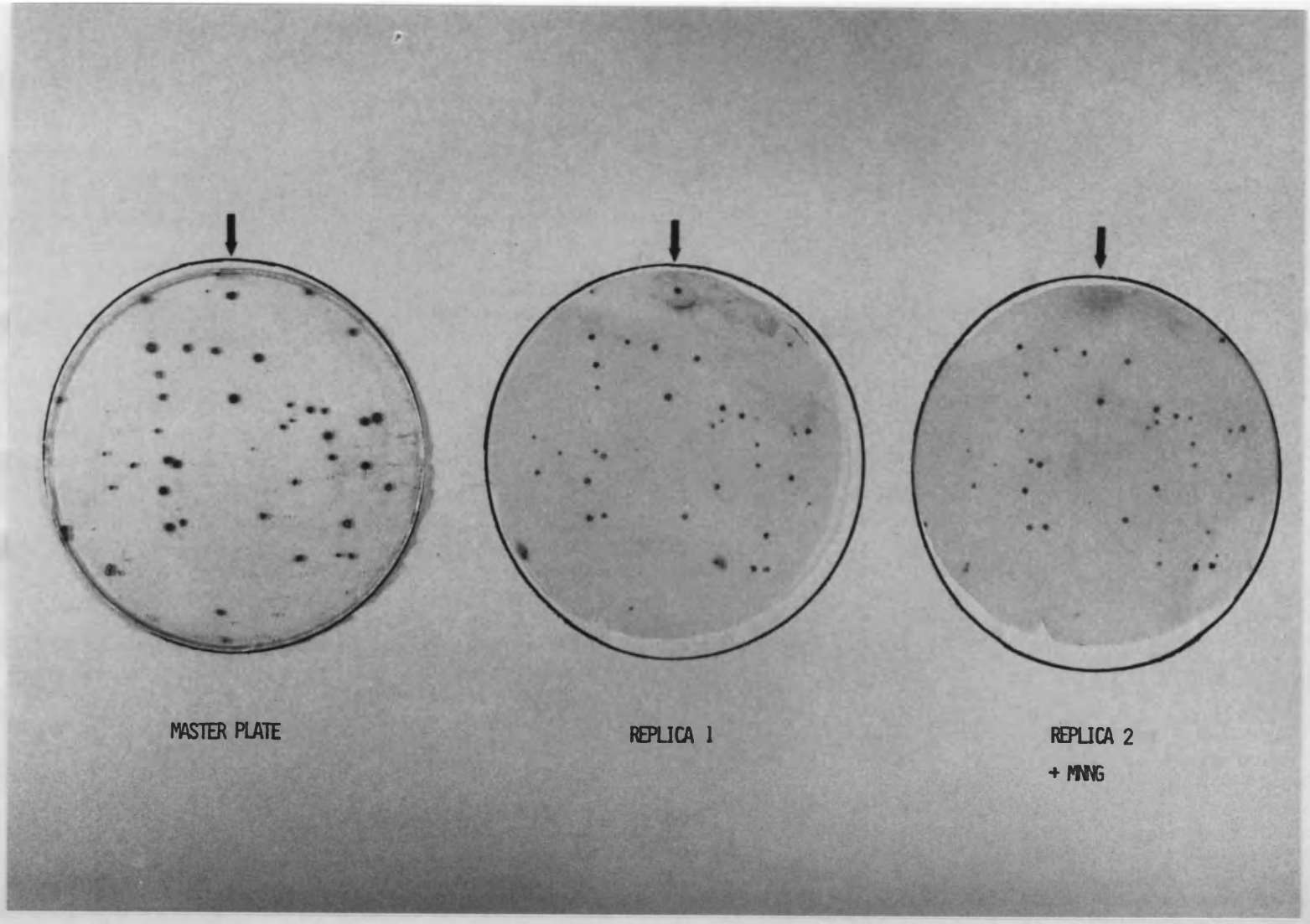


Plate 5.1. Isolation of the MNNG-sensitive clone CHO-K1S by replica plating on polyester mesh discs. The colony missing from replica 2, following a dose of 0.02 $\mu g ml^{-1}$ MNNG, was isolated from the corresponding position on a master replica disc.

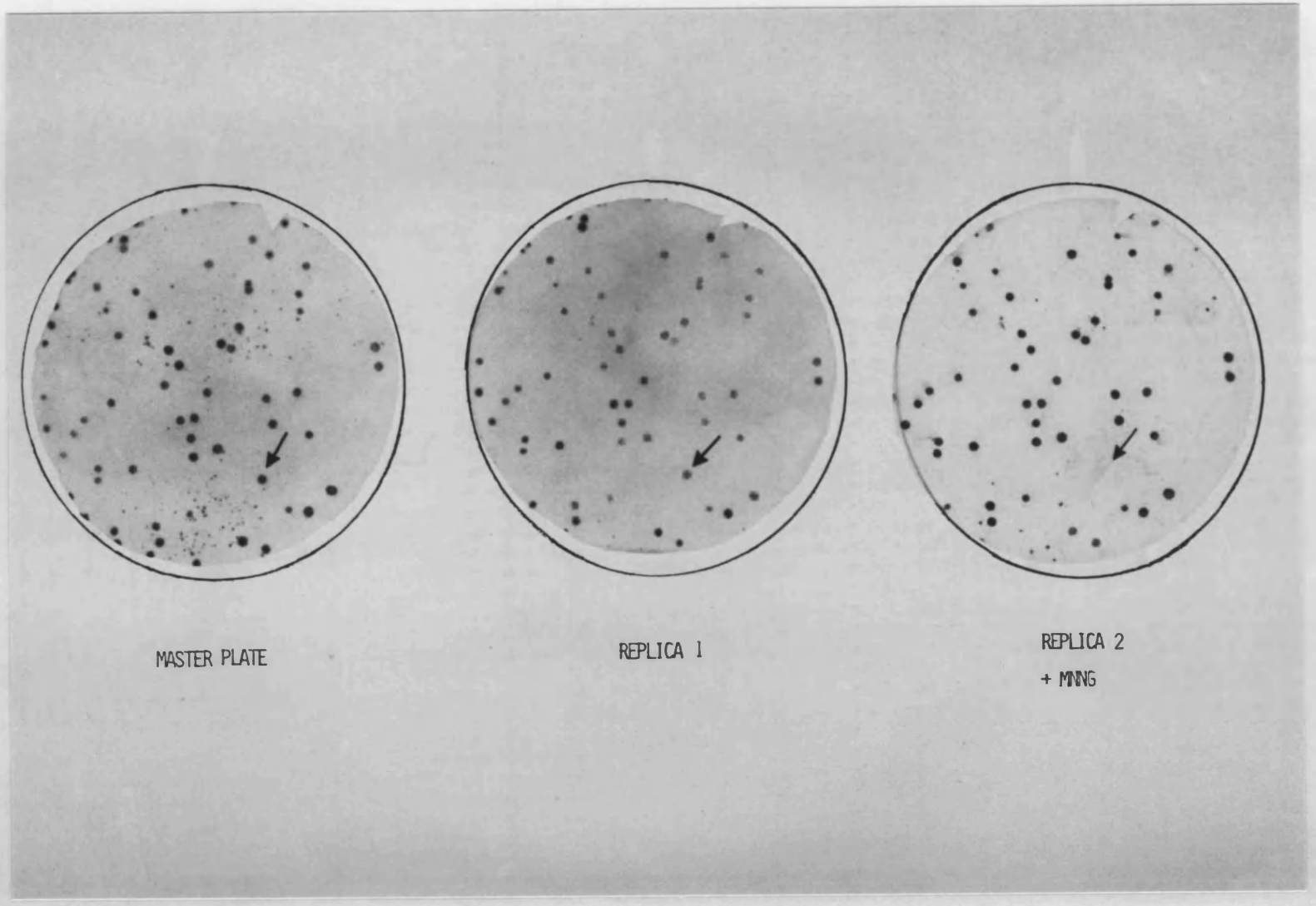


Plate 5.2. Isolation of the MNNG-sensitive clone V79-379AS by replica plating on polyester mesh discs. The colony missing from replica 2, following a dose of 0.02 $\mu g ml^{-1}$ MNNG, was isolated from the corresponding position on a master replica disc.

an MNNG dose of 0.02 $\mu g ml^{-1}$).

5.4. The effect of adaptive pre-treatment on the MNNG-induced cytotoxicity of the sensitive and resistant cell variants.

5.4.1. Toxic response tests : single plated CHO-K1S, CHO-K1R, V79-379AS and V79-379AR cells.

After isolation of each cell variant it was first necessary to establish if the selection procedures had been effective. Therefore MNNG dose-response curves were constructed for each variant line.

Two days prior to the experiments cultures of each cell line were started by inoculating $5x10^5$ cells into 150ml culture bottles each containing 15ml of Hams F10 + 5% FCS, gassed with 5% CO_2 in air and incubared at 37.5°C. After 46-48 hours growth cell suspensions were obtained and the cell density determined. The suspensions were serially diluted to densities between $1x10^3$ and $8x10^4$ cell ml^{-1}, 0.1ml aliquots of each suspension were transfered to 50mm T/C petri dishes containing 4.9ml of pre-warmed Hams F10 + 5% FCS, to give between 100 and 8000 cells/plate, depending on the expected cytotoxicity. After 3 hours incubation the cells were exposed to MNNG at concentrations in the range 0.02 to 0.3 $\mu g ml^{-1}$. Following a 2 hour exposure the medium was replaced with 5ml fresh, pre-warmed Hams F10 + 5% FCS and the cells re-incubated for 7 days, stained, coded and scored 'blind'.

MNNG-induced dose-response curves for each cloned line are shown in Figs.5.1. and 5.2. The values for CHO-K1S and V79-379AS are expressed as the mean survival ± standard error, of three independent experiments, plotted on a log scale against MNNG concentration. The values presented for CHO-K1R and V79-379AR cells, however, show data derived from four independent

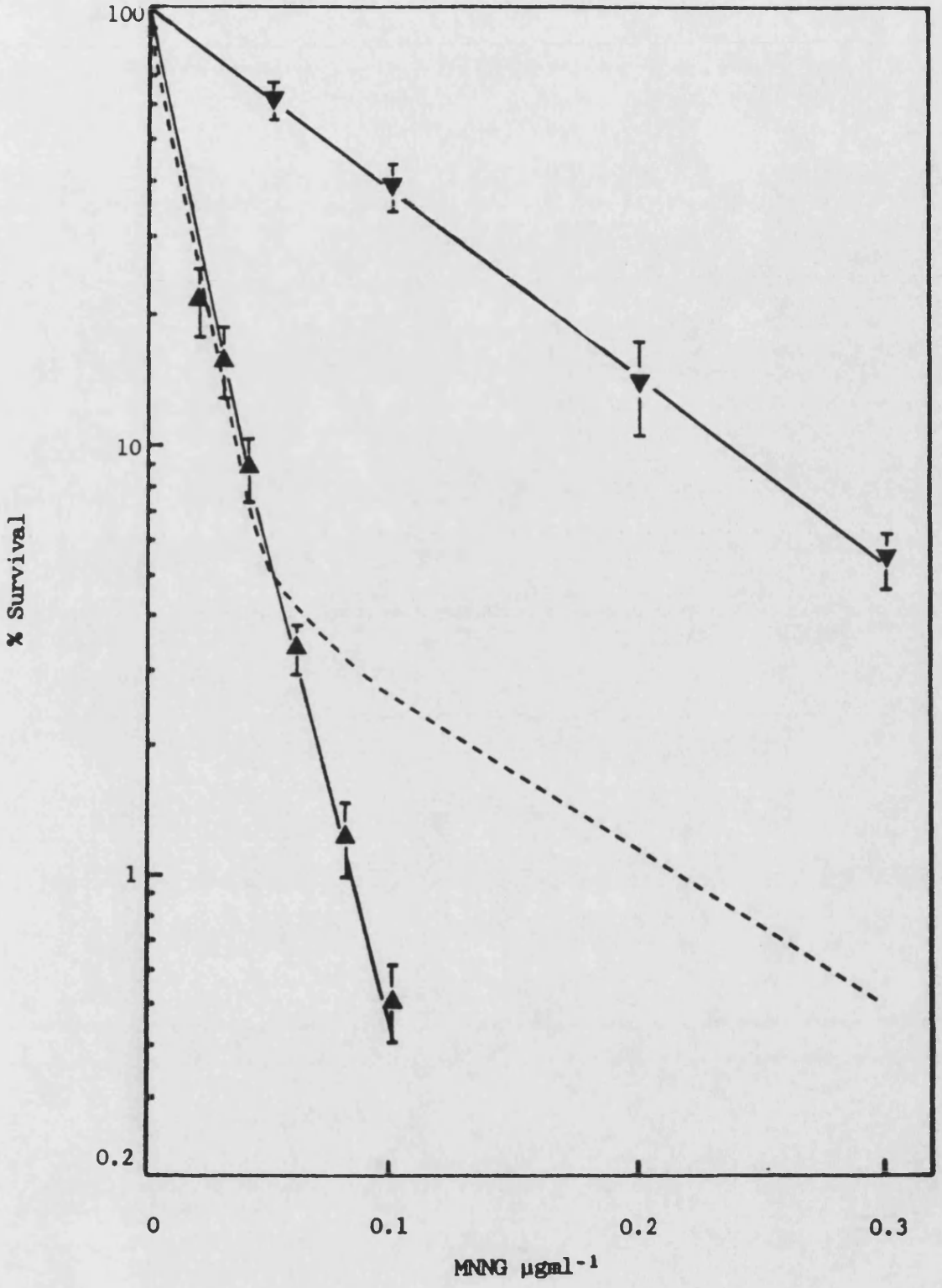


Figure 5.1. Dose-response curve for CHO-K1S (▲) and CHO-K1R cells (▼) grown in Hams F10 + 5% FCS, treated with MNNG (▲ , n=3 ± S.E.; ▼ , n=4 ± S.E.). The dose-response of CHO-K1 cells (-----) has been included for comparative purposes.

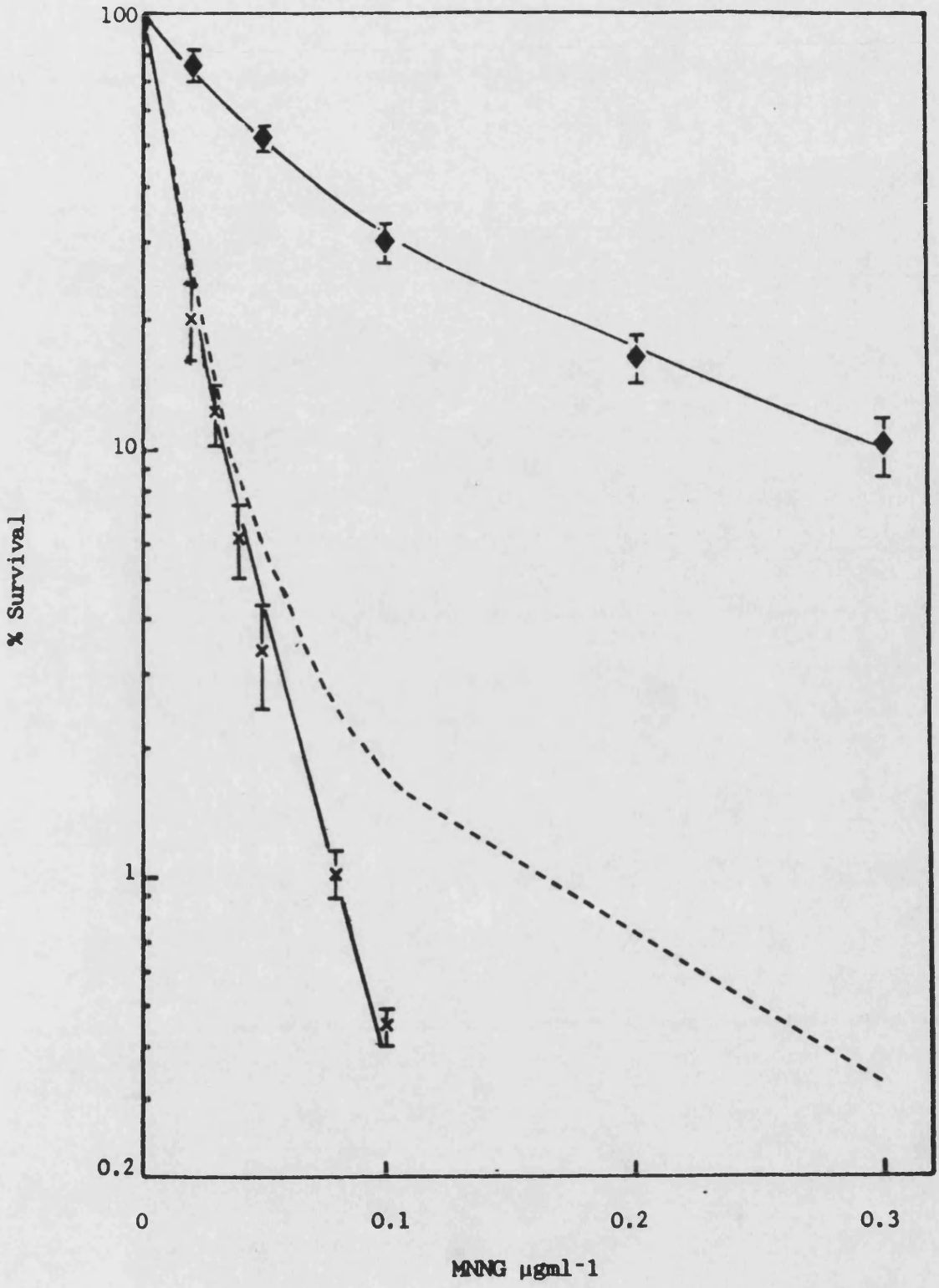


Figure 5.2. Dose-response curve for V79-379AS cells (×) and V79-379AR cells (◆) grown in Hams F10 + 5% FCS, treated with MNNG (× , n=3 ± S.E.; ◆ , n=4 ± S.E.). The dose-response curve of V79-379A cells (-----) has been included for comparative purposes.

experiments. The parental dose-survivor curves are also shown in Figs.5.1. and 5.2., for comparative purposes, and are represented by single broken lines derived from the data presented in Figs.3.2. and 3.3.

The MNNG-sensitive cell clones CHO-K1S and V79-379AS exhibited cell killing identical to the parental lines up to a dose level of 0.05 $\mu g ml^{-1}$. Above this level the cells appear to be much more sensitive to MNNG than their respective parental lines. D_{37} values for parental and sensitive cell are shown below in Table 5.1.

Table 5.1. A comparison of the relative sensitivities of different cell types to MNNG, as measured by the D_{37} values.

Cell line.	D_{37} value $\mu g ml^{-1}$.
CHO-K1	0.021
CHO-K1S	0.018
CHO-K1R	0.110
V79-379A	0.014
V79-379AS	0.016
V79-379AR	0.070

This suggests that the replica plating procedure was successful resulting in the isolation of a cell line more MNNG-sensitive than the parental line.

Resistant cell variants CHO-K1R and V79-379AR were found to be 5.2 and 5.7 fold more resistant to the cytotoxic action of MNNG when compared to their respectrive parental lines shown above in Table 5.1.

Both lines were maintained in the absence of MNNG for two months without a loss of MNNG resistance.

Resistant clones previously isolated by other workers, have shown between 3.3 and 40 fold greater resistances to mutagen challenge by the selecting mutagen, when compared to their parental lines (Friedman and Huberman, 1980; Goldmacher *et al.*, 1986; Goth-Goldstein, 1987; Ishida and Takahashi, 1987).

5.4.2. The effect of adaptive pre-treatment on the MNNG-induced cytotoxicity of CHO-K1R and V79-379AR cells.

Cultures of each cell line were set up and pre-treated as previously described (3.4.2.). Construction of MNNG dose-response curves for CHO-K1S and V79-379AS cells was attempted many times with little success. Colonies were not visible even after 11 days incubation, an observation probably explained by the results reported in 5.5.2.

It was possible, however, to construct MNNG dose-response curves for both CHO-K1R and V79-379AR lines following adaptive pre-treatment (Fig.5.3.). CHO-K1R cells exhibited no change following adaptive pre-treatment with control and pre-treated D_{37} values of 0.11 $\mu g ml^{-1}$. In contrast, pre-treated V79-379AR cells showed a higher D_{37} value than that of control untreated V79-379AR cells i.e. 0.13 and 0.07 $\mu g ml^{-1}$ respectively. Furthermore survival of pre-treated cells is seen to be an exponential function of dose, rather than a bi-phasic relationship as seen for untreated

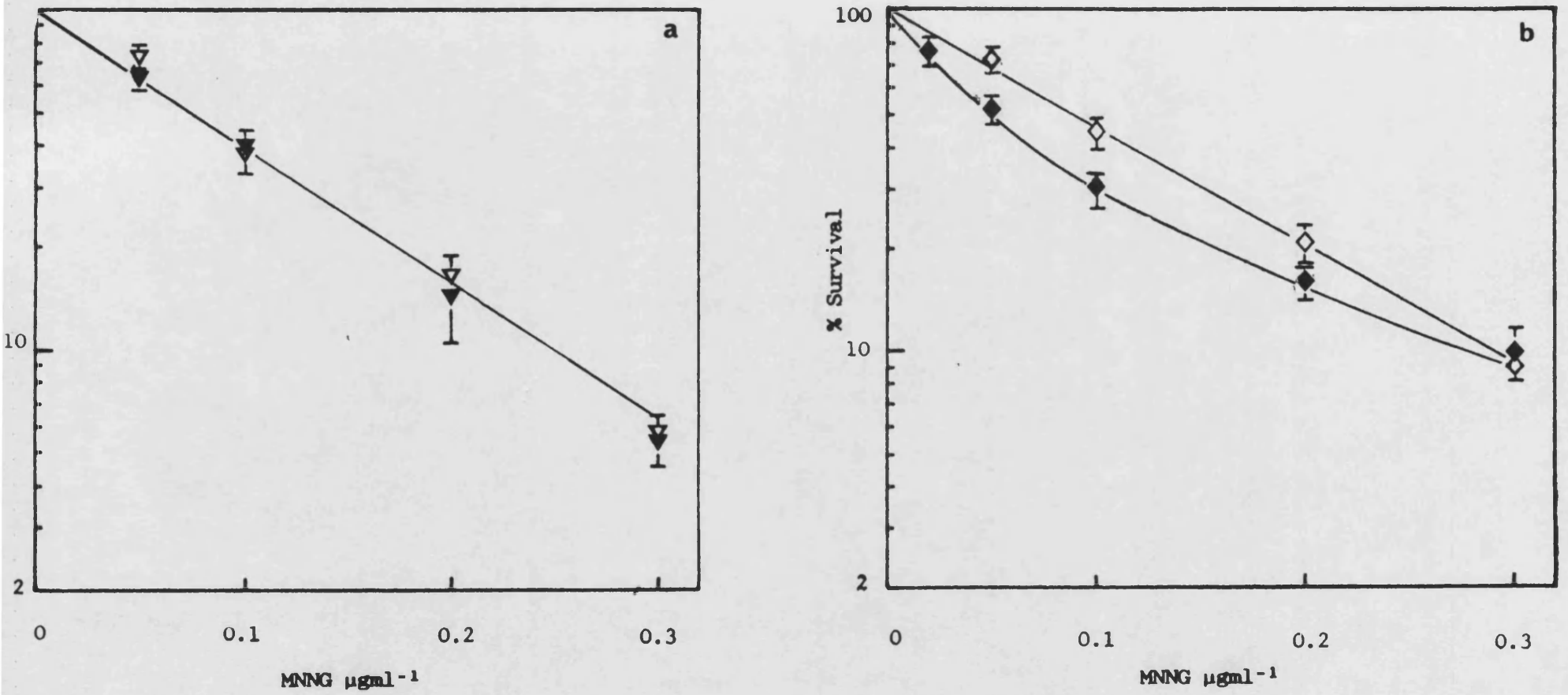


Figure 5.3. The effect of adaptive pre-treatment, by Protocol 2, on the dose-response curves of CHO-K1R cells, (a), and V79-379AR cells, (b), grown in Hams F10 + 5% FCS. Open and closed symbols represent pre-treated cells and control cells respectively (n=3 ± S.E.).

V79-379AR cells.

5.5. The effect of adaptive pre-treatment on the growth parameters of the isolated clones.

5.5.1. Determination of the growth parameters of CHO-K1S, CHO-K1R, V79-379AS and V79-379AR cells.

Growth curves for the above cells were constructed as described in 4.3.1. and are shown in Figures 5.4. and 5.5. The growth parameters derived from these data are shown on Table 5.2.

CHO-K1S cells show growth parameters that are identical to the parental cell line both having a population doubling times of 14.4 hours and lag times of 6 hours (Tables 4.2. and 5.2.). V79-379AS cells exhibit the same lag time (6 hours) as their parental cells but have a population doubling time of 14.4 hours compared to 11.4 hours (Tables 4.2. and 5.2.). However, this value of T lies within the previously reported range of 10-16 hours for V79 cells (Bradley *et al.*, 1981; Hsie *et al.*, 1981; Kao and Puck, 1974) and is therfore considered to be no different to control.

CHO-K1R cells exhibited increased lag and population doubling times compared to parental cells (Tables 4.2. and 5.2.). These values are similar to those seen for parental cells following adaptive pre-treatment where both lag and doubling times are increased (Table 4.3.). This suggests that adaptive pre-treatment may have enriched the parental population with resistant cells so mimicing the resistant clone selection procedure. This theory, however, may not hold true for V79-379A cells since resistant cells show increased lag and doubling times, over parental, but pre-treated parental cells show no increase of either lag or doubling times (Tables 4.3. and 5.2.).

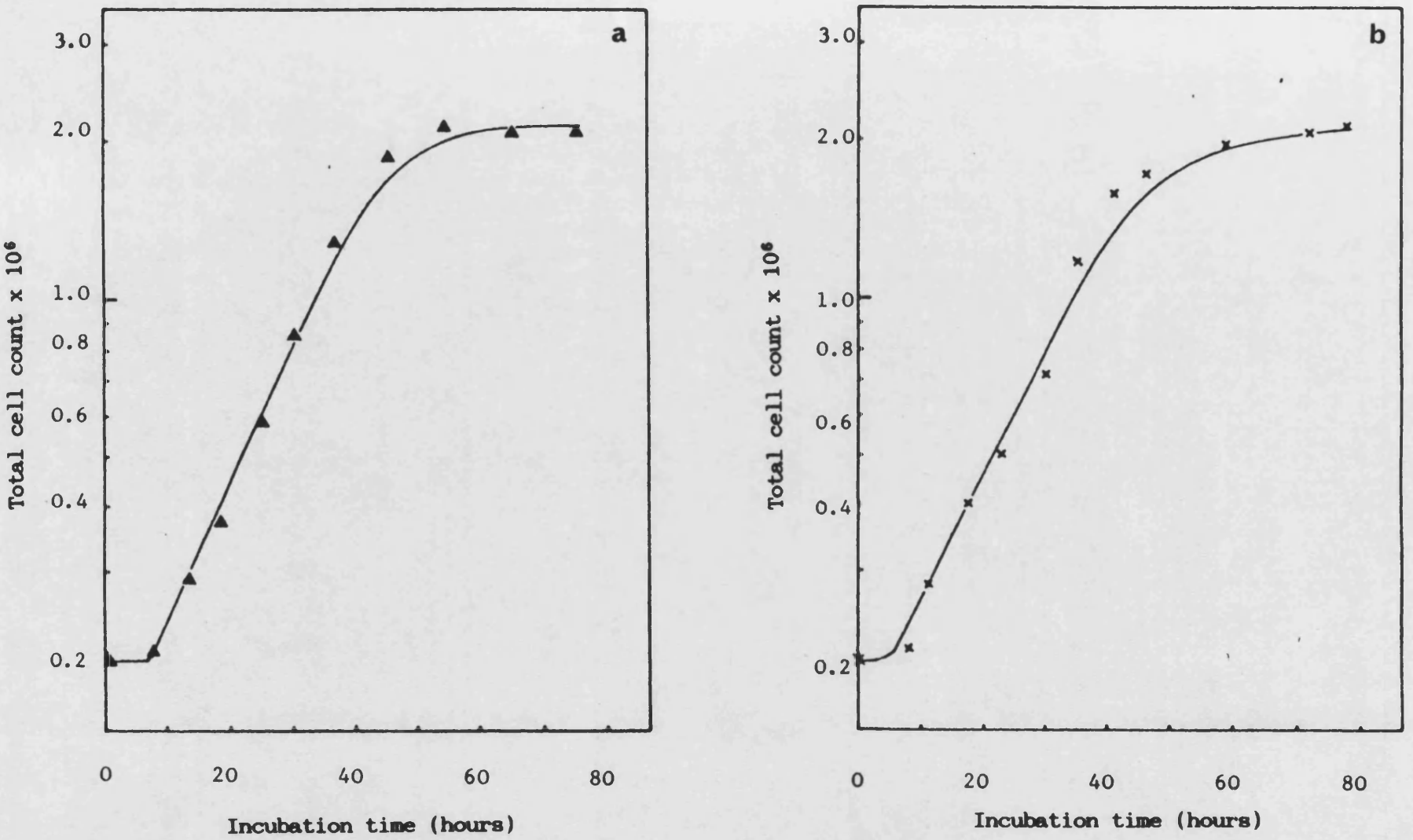


Figure 5.4. Growth curves of CHO-K1S cells, (a), and V79-379AS cells, (b), grown in Hams F10 + 5% FCS.

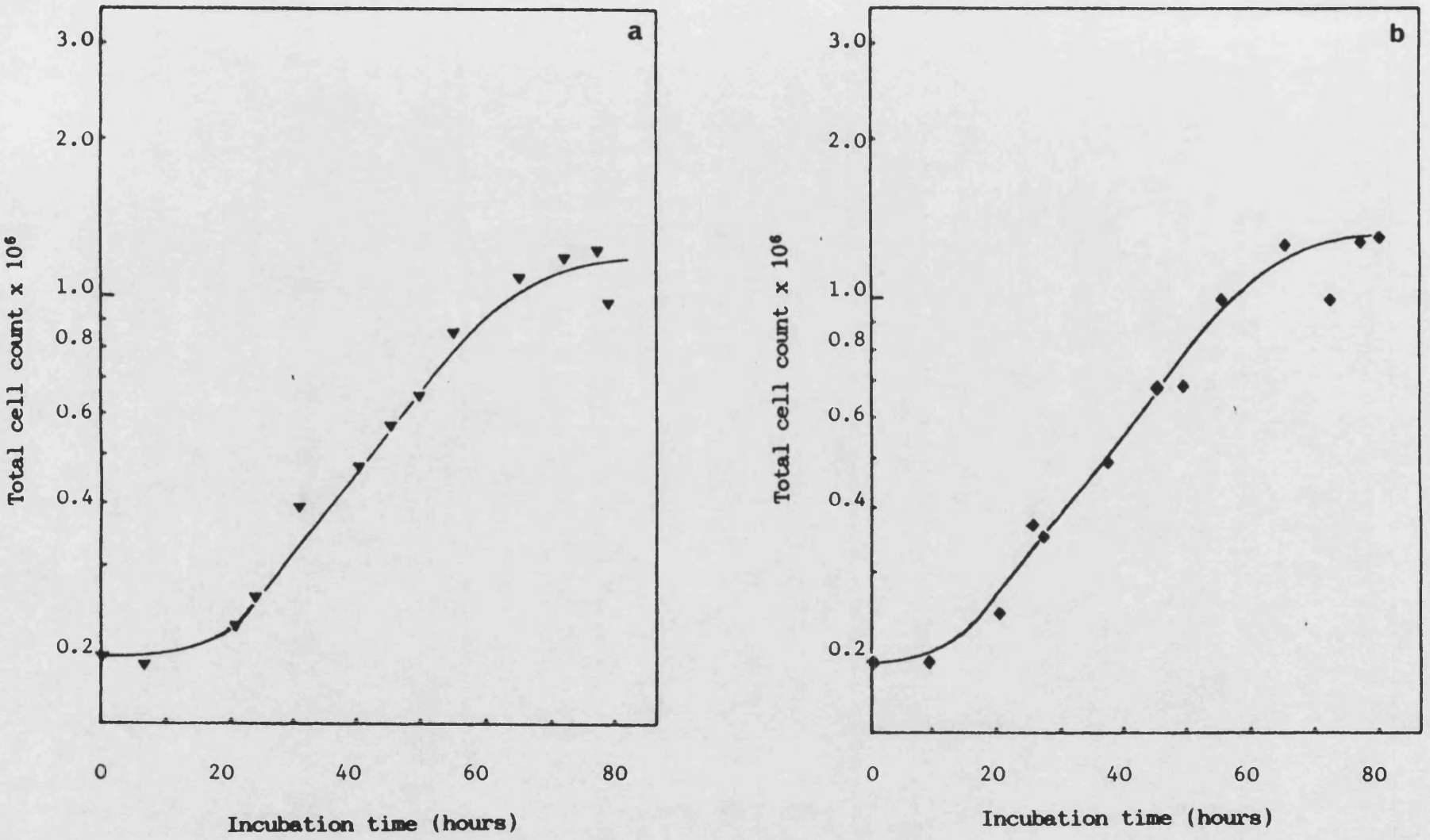


Figure 5.5. Growth curves of CHO-K1R cells, (a), and V79-379AR cells, (b), grown in Hams F10 + 5% FCS.

Table 5.2. Growth parameters of CHO-K1S, CHO-K1R, V79-379AS and V79-379AR cells grown in Hams F10 + 5% FCS.

Cell line	Lag time (hrs)	k value (hrs^{-1})	T (hrs)	r
CHO-K1S	6	0.0695	14.4	0.97
CHO-K1R	17.5	0.0382	26.1	0.99
V79-379AS	6	0.0679	14.7	0.99
V79-379AR	11.5	0.0409	24.4	0.98

k = Exponential growth rate constant.
T = Population doubling time.
r = Correlation coefficient.

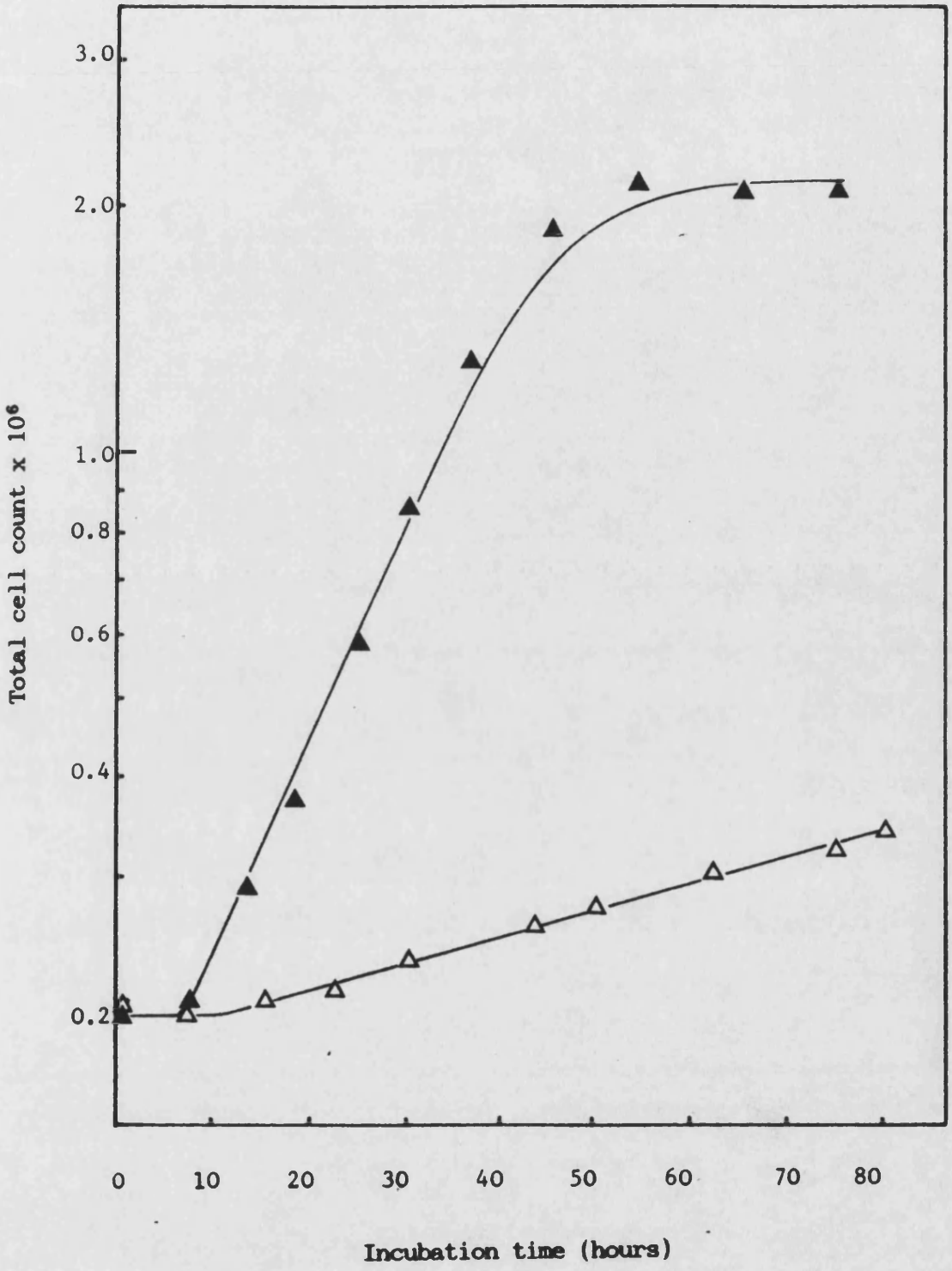


Figure 5.6. The effect of adaptive pre-treatment, by Protocol 2, on the growth of CHO-K1S grown in Hams F10 + 5% FCS. ((△) = pre-treated cells; (▲) = control cells.)

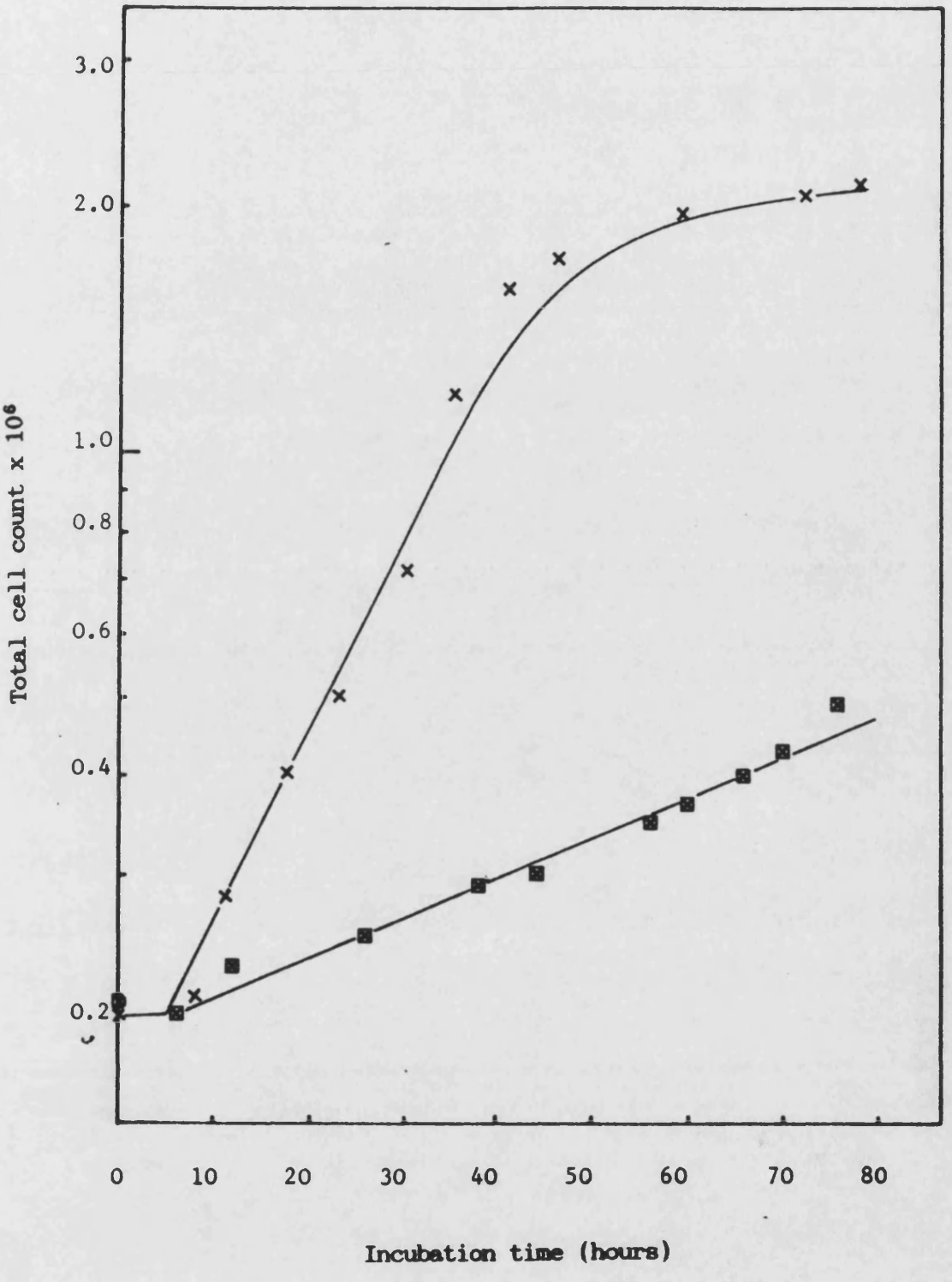


Figure 5.7. The effect of adaptive pre-treatment, by Protocol 2 on the growth of V79-379AS cells in Hams F10 + 5% FCS. ((■) = pre-treated cells; (×) = control cells.)

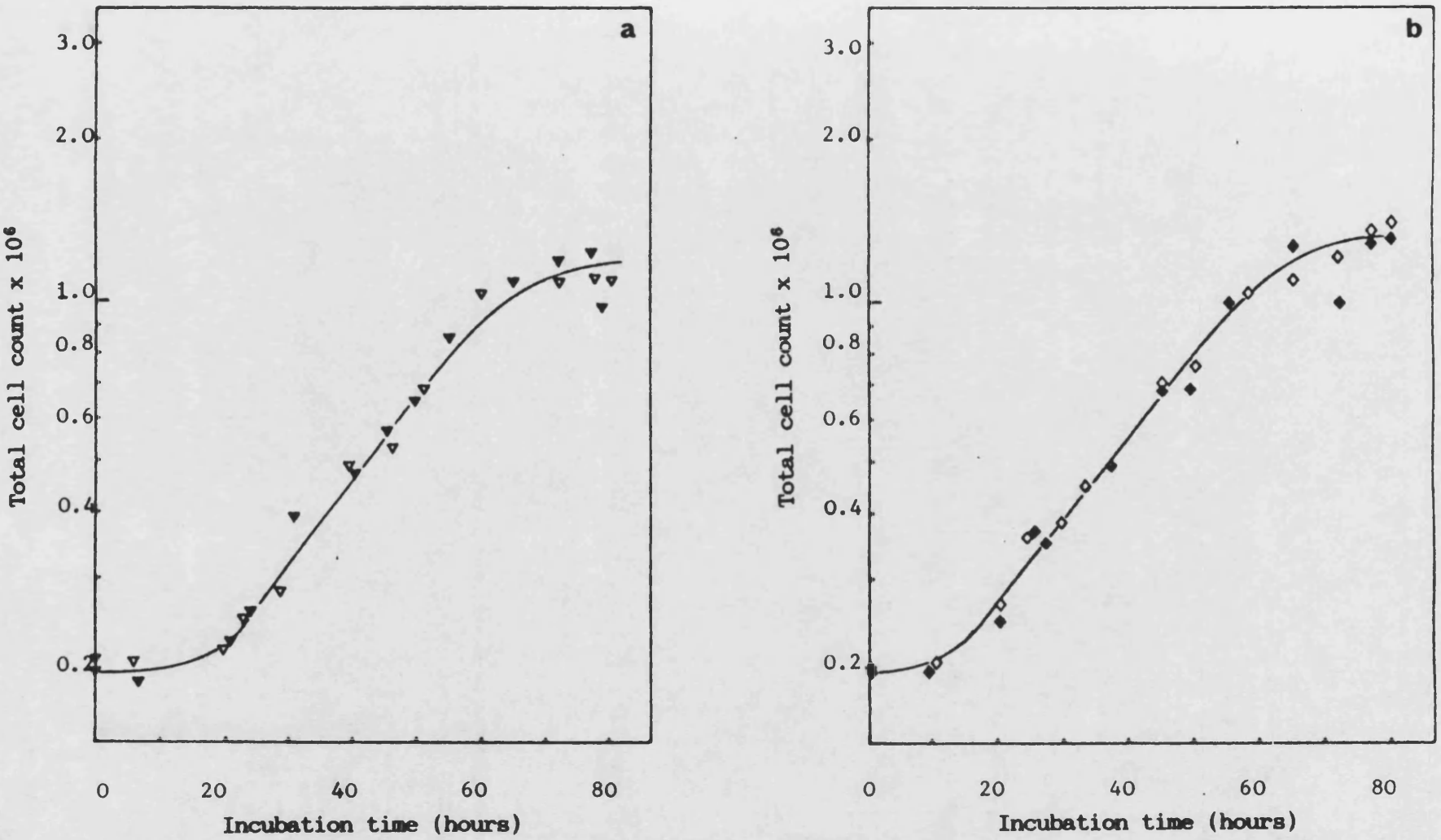


Figure 5.8. The effect of adaptive pre-treatment, by Protocol 2, on the growth of CHO-K1R cells, (a), and V79-379AR cells, (b), grown in Hams F10 + 5% FCS. Open and closed symbols represented pre-treated and control cells respectively.

Table 5.3. The effect of adaptive pre-treatment, by Protocol 2 on the growth parameters of CHO-K1S, CHO-K1R, V79-379AS and V79-379AR cells grown in Hams F10 + 5% FCS.

Cell line	Lag time (hrs)	k value (hrs^{-1})	T (hrs)	r
CHO-K1S	6	0.0695	14.4	0.97
CHO-K1S + ADA	10	0.00922	108.5	0.99
CHO-K1R	17.5	0.0382	26.1	0.99
CHO-K1R + ADA	18	0.0386	25.9	0.99
V79-379AS	6	0.0679	14.7	0.99
V79-379AS + ADA	6	0.0146	68.4	0.98
V79-379AR	11.5	0.0409	24.4	0.98
V79-379AR + ADA	11.5	0.0409	24.4	0.99

k = Exponential growth rate constant. T = Population doubling time.
ADA = adaptive pre-treatment by Protocol 2.

5.5.2. The effect of adaptive MNNG pre-treatment on the growth paramaters of CHO-K1S, CHO-K1R, V79-379AS and V79-379AR cells.

To determine the effect of adaptive pre-treatment on the growth parameters of these cell lines, cultures were set up and pre-treated with repeated doses of MNNG as detailed in 3.4.2. and Fig.4.14. At t=0 the cells were dispersed with trypsin and the cell density determined. $2x10^5$ cells of each cell line were inoculated into a number of $25cm^2$ T/C flasks containing 5ml of Hams F10 + 5% FCS. Growth curves for each cell line, with or without adaptive pre-treatment, were constructed as described in 4.3.2. and the results are presented in Figs.5.6., 5.7 and 5.8., and also on Table 5.3.

Following adaptive pre-treatment the population doubling times of both MNNG-sensitive cell lines were much greater than untreated cells with CHO-K1S cells having a doubling time of 108.5 hours and V79-379AS a value of 68.4 hours (Table 5.3.). Untreated cells had T values of 14.4 and 14.7 hours respectively (Table 4.2).

5.6. The MNNG-induced mutation to Oua^R of the isolated sensitive or resistant clones.

5.6.1. Determination of the induced mutation to Oua^R of CHO-K1S, CHO-K1R, V79-379AS and V79-379AR cells.

Two days prior to the experiment, duplicate cultures of each cell line were set up by inoculating $5x10^5$ cells into 150ml culture bottles each containing 15ml Hams F10 + 5% FCS. The cultures were gassed with 5% CO_2 in air and incubated at

37.5°C. After 46-48 hours growth the cells were dispersed with trypsin and the cell density determined. These suspensions were used to inoculate $2x10^5$ cells into 90mm T/C petri dishes (five dishes per mutagen treatment i.e. $1x10^6$ cells in total) containing Hams F10 + 5% FCS to 10ml (selection plates). Further dilution of the initial suspension allowed survival plates to be set up in parallel as outlined in 3.2.1. and 3.2.2. Following a 3 hour incubation, MNNG doses in the range 0.02 to 0.3 μgml^{-1} were added to both survival and selection plates. After 2 hours mutagen treatment the culture medium was replaced with fresh, pre-warmed Hams F10 + 5% FCS and the plates re-incubated. Following a further 46 hour incubation, (mutant expression time, Fig.4.1.), the medium in the selection plates was replaced with 9ml Hams F10 + 5% FCS plus 1ml of stock ouabain solution (2.4.2.). All plates were incubated and then stained, coded and scored 'blind' after 7 days (survival plates) and 8 days (selection plates). The results of these experiments are illustrated as a plot of induced mutants per 10^6 survivors, on a log scale, against percentage survival, on a log scale, in Fig.5.9.

At each MNNG dose level CHO-K1S and V79-379AS cells show a greater number of induced mutants than those of their respective parental lines. When mutation is considered as a function of cell survival, however, the these sensitive clones appear to be as equally mutable as their parental lines (Fig.5.9.). CHO-K1R and V79-379AR cells show the opposite i.e. a decreased number of mutants at all MNNG dose levels, but again these cells are seen to be as equally mutable as the parental lines when plotted as a function of cell survival (Fig.5.9.).

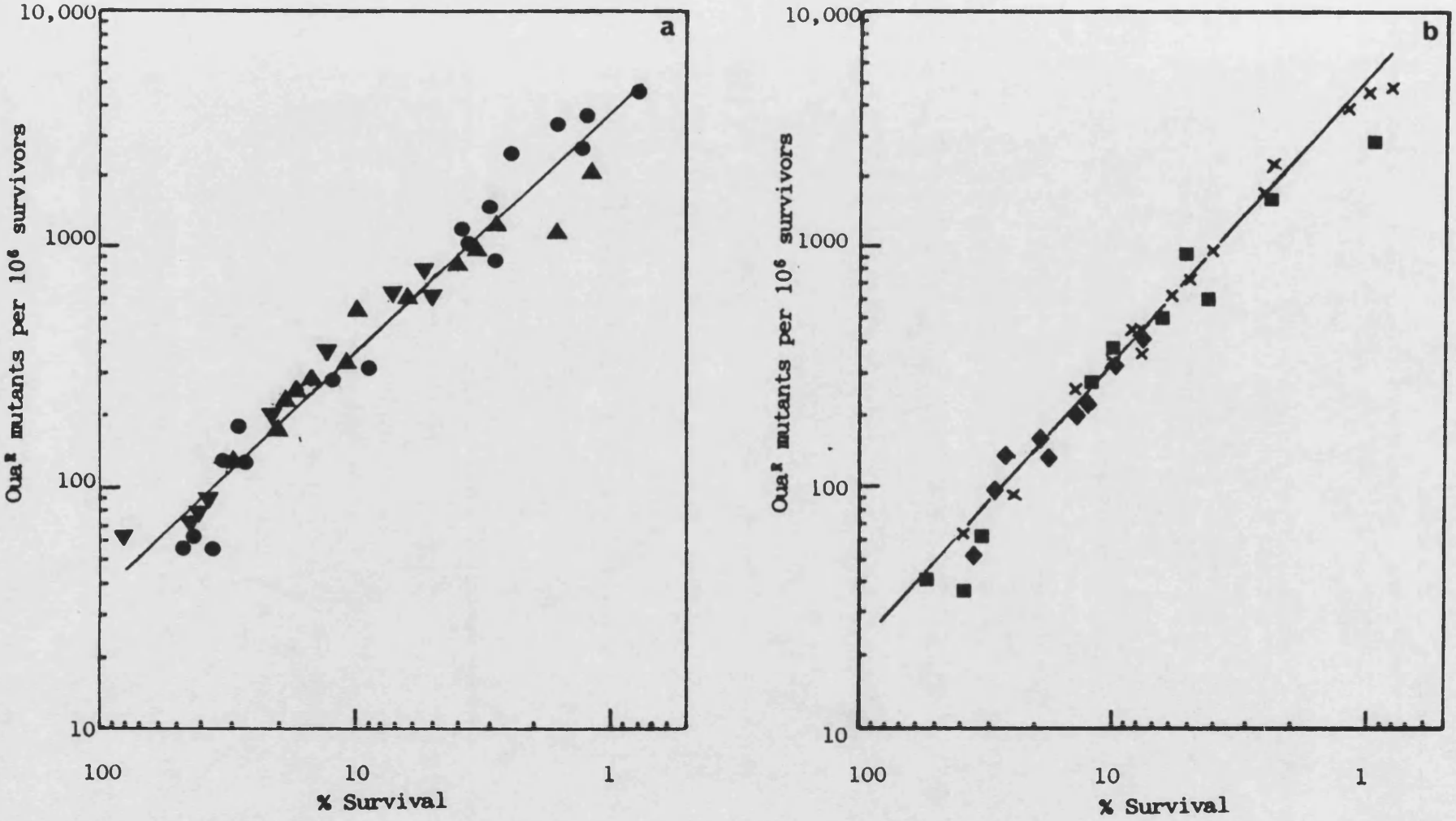


Figure 5.9. The relationship between the MNNG-induced mutation frequency to Oua^R and percentage survival of CHO-K1 (●), CHO-K1S (▲), CHO-K1R (▼), V79-379A (■), V79-379AS (×) and V79-379AR cells (◆) grown in Hams F10 + 5% FCS.

5.6.2. The effect of adaptive pre-treatment on the MNNG-induced mutation to Oua^R of CHO-K1R and V79-379AR cells.

It was shown in 4.4.1. that adaptive pre-treatment severely retarded the growth of CHO-K1S and V79-379AS cells. Therefore it was decided to determine the effect of pretreatment on the MNNG-induced mutation to Oua^R in the resistant clones CHO-K1R and V79-379AR only.

Two days prior to the experiment, duplicate cultures of each cell line were started by inoculating $5x10^5$ cells into 150ml culture bottles each containing 15ml Hams F10 + 5% FCS. The cultures were gassed with 5% CO_2 in air and incubated at 37.5°C. After 46-48 hours growth the cells were dispersed with trypsin and the cell density determined. Adaptive pre-treatment was performed as detailed in 3.4.2. At t=0 selection and survival plates were set up and the procedure outlined in 5.6.1. followed through. Adaptive pre-treatment appears to have little effect on the induced mutation frequencies of either CHO-K1R or V79-379AR cells when compared to the values obtained for untreated resistant cells or those of their respective parental cells (Fig.5.10.). The spontaneous mutation frequencies to Oua^R are not altered by pre-treatment as previously observed following adaptive pre-treatment of CHO-K1 and V79-379A cells (4.2.2.).

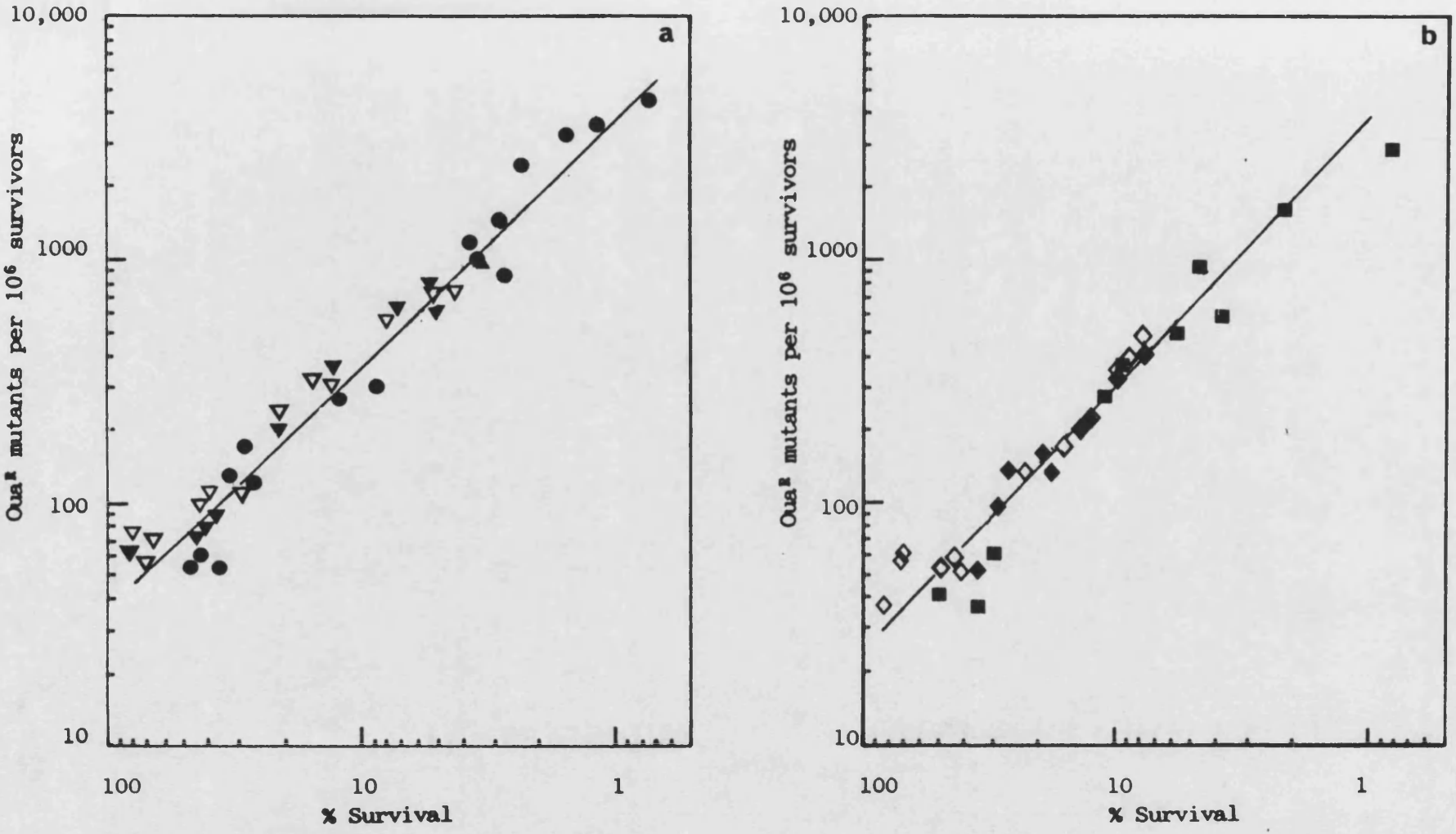


Figure 5.10. The effect of adaptive pre-treatment, by protocol 2, on the relationship between MNNG-induced mutation frequency to Oua^R and percentage survival of CHO-K1R cells and V79-379AR cells grown in Hams F10 + 5% FCS. ((●) = CHO-K1; (▼) = CHO-K1R; (▽) = CHO-K1R following adaptive pre-treatment; (■) = V79-379A; (◆) = V79-379AR; (◇) = V79-379AR following adaptive pre-treatment).

5.7. Conclusions.

From the results reported in this chapter the following can be concluded:

1) It was possible to separate two discrete sub-populations of both CHO-K1 and V79-379A cells by virtue of their differing sensitivities to MNNG.
2) Cells surviving MNNG doses of 0.3 $\mu g ml^{-1}$ were isolated by ring cloning and were found to have a greater resistance to MNNG-induced cell killing than their respective parental line (Figs.5.1. and 5.2.). CHO-K1R and V79-379AR cells had D_{37} values of 0.11 and 0.07 $\mu g ml^{-1}$ respectively (5.4.1.). These values are 5.2 and 5.7 fold greater than their respective parental lines (Table 5.1.).
3) MNNG-induced dose-survivor curves of the derived MNNG-sensitive clones CHO-K1S and V79-379AS were identical to the parental survival up to a dose level of 0.05 $\mu g ml^{-1}$, beyond which the cells exhibited a much greater MNNG-sensitivity (Figs.5.1. and 5.2.).
4) Survival of CHO-K1R cells was seen to be an exponential function of MNNG dose which was unaffected by adaptive pre-treatment (Fig.5.3.). The slightly bi-phasic nature of the V79-379AR dose-response curve was altered to a simple exponential decrease in cell survival by, with increase in MNNG dose, adaptive pre-treatment (Fig.5.3.). An increase in D_{37} value to 0.13 $\mu g ml^{-1}$ compared to 0.07 $\mu g ml^{-1}$ for untreated V79-379AR cells was seen.
5) CHO-K1R and V79-379AR cells exhibited population doubling times of 26.1 and 24.4 hours with lag times of 17.5 and 11.5 hours respectively (Fig.5.5.; Table 5.2.). These parameters

were unaffected by adaptive pre-treatment (Fig.5.8.; Table 5.3.).

6) CHO-K1S and V79-379AS cells exhibited population doubling times of 14.4 and 14.7 hours respectively. Both had lag times of 6 hours (Fig.5.4.; Table 5.2.). These parameters are comparable to those of parental cells (Table 4.2.). Population doubling times (T) are dramatically increased following adaptive pre-treatment. T for CHO-K1S was increased by 94.1 hours, from 14.4 to 108.5 hours. For V79-379AR, T was increased by 53.7 hours, from 14.7 to 68.4 hours (Figs.5.6. and 5.7.; Table 5.3.). The lag time of CHO-K1S was increased by 4 hours, from 6 to 10 hours, following pre-treatment. The lag of V79-379AS cells was unchanged at 6 hours, following pre-treatment (Table 5.3.).

7) CHO-K1S and V79-379AS cells exhibited induced mutation frequencies to Oua^R that were higher, on a dose basis, than those of parental cells but when analysed at equicytotoxic doses these cells were seen to be as equally mutable as their respective parental cell lines (Fig.5.9.).

8) CHO-K1R and V79-379AR cells showed MNNG-induced mutation frequencies to Oua^R that were lower, on a dose basis, than those of parental cells. Again, analysis of the data at equicytotoxic doses revealed these cells to be as equally mutable as their respective parental cell lines (Fig.5.9.). Mutation frequencies of each resistant line were unaltered by adaptive pre-treatment (Table.5.10.). Spontaneous (vehicle control) mutation frequencies remained low following pre-treatment (cf. 4.2.3.).

These conclusions are discussed in chapter 8.

CHAPTER 6. ANALYSIS OF O^6-METHYLGUANINE-DNA-METHYLTRANSFERASE ACTIVITY IN CHO-K1, CHO-K1R, V79-379A AND V79-379AR CELLS.

6.1. Introduction.

The bacterial adaptive response essentially involves two repair enzymes (1.2.), whose activity can be induced by mutagen treatment at sub-lethal concentrations. There is conflicting evidence for the presence of these enzymes in mammalian cells (1.4.1. and 1.4.2.). Interests, in the main, have been focused on the O^6-methylguanine-DNA-methyltransferase (O^6MGMT) enzyme since this de-methylates the most important pre-mutagenic lesion O^6-methyl guanine (O^6MG) leaving the natural base guanine (Beranek *et al.*, 1983; Newbold *et al.*, 1980). CHO and V79 cells have been reported to have a Mer$^-$ phenotype i.e. methyl exision repair deficient (Foote *et al.*, 1983; Foote and Mitra, 1984; Yarosh *et al.*, 1983, 1984) and so are unable to remove the O^6MG lesion. If this is so, the apparent reduction of induced mutation frequencies following adaptive pre-treatment, observed for CHO-K1 and V79-379A cells (4.2.2.), is mediated via a mechanism not involving O^6MGMT. Waldstein *et al.*, (1982c) found constitutive levels of O^6MGMT in both CHO and V79 cells suggesting a possible involvement of this enzyme. However, this evidence was disputed by a number of authors (Foote *et al.*, 1983; Foote and Mitra 1984; Harris *et al.*, 1983; Yarosh *et al.*, 1983, 1984).

To elucidate this problem it was decided to determine whether CHO-K1 and V79-379A cell lines actually possess the O^6MGMT enzyme, and if so is it mutagen inducible. This was carried out by incubation of cell free extracts of each cell line with alkylated DNA and assessing the disappearance of O^6MG using a HPLC

separation technique. The O^6MG removal ability of pre-treated parental cell extracts and that of the resistant cell variants, also shown to have low induced mutation frequencies (5.6.1.), were determined. The MNNG-sensitive clones CHO-K1S and V79-379AS were not assayed for their ability to de-methylate O^6MG since the population doubling times of both lines was drastically increased by adaptive pr-treatment, making experimentation lenghty and unreliable. A cell line known to be Mer^+ i.e. $C3H10T^1/_2$ (Grisham and Smith, 1984; Smith *et al.*, 1981) was used as a positive control and also to validate the assay procedure.

6.2. Materials.

This section includes materials not mentioned in Chapter 2.

6.2.1. Mobile phase.

Three mobile phase systems were used during the course of these studies;

Mobile phase A : 5% methanol (HPLC grade, FSH Ltd., Loughborough): 95% H_2O, 6.5m $NH_4H_2PO_4$ (BDH ChemicalsLtd, Poole), pH=5.0.

Mobile phase B : 40% methanol : 60% H_2O, 6.5M $NH_4H_2PO_4$, pH=5.0.

Mobile phase C : 70% methanol : 30% H_2O.

Distilled water, used for the preparation of all mobile phase systems, was made free of particulate matter by vacuum filtration through a Millipore filtration unit fitted with a 47mm x 0.45 µm pore membrane filter. De-gassing was achieved by bubbling a constant stream of Helium through the mobile phase.

6.2.2. Column and HPLC equipment.

Separation of methylated nucleic acid bases was carried out using a reversed-phase 250 mm x 4.9 mm i.d. ODS-hypersil (5 µm particle size) column, pre-packed by Hi-Chrom Ltd., Reading,

The Spectra-Physics auto-analyser system was used in these studies. This comprised an SP8100 chromatograph with autosampler connected to a SP8440 variable wavelength detector both interfaced to a SP4200 dual channel computing integrator. The SP8100, SP8440 and SP4200 are interfaced in parallel and controlled by a Basic program running on an ADDS terminal operated by a Viewpoint/60 keyboard.

6.2.3. Column test system.

It is recommended by the manufacturers, that the performance of columns is tested on arrival and periodically during column use. The column efficiency is normally defined in terms of the number of theoretical plates, N, and is expressed as plates per column or plates per metre. It is calculated from either of the following equation:

$$N = 5.54(t_a/W^1/_2)^2 \qquad \text{.....Equation 6.1.}$$

and is illustrated in Figure 6.1.

Column testing is necessary to monitor deteriorations in performance as the life of the column increases and to eliminate these as the cause of poor separations. The standard test mixture for ODS-hypersil columns was Benzamide, Acetophenone and Benzophenone as recommended by Shandon Ltd, the manufacturers of this type of packing material. Fig.6.2. shows a typical column test chromatogram carried out before each experimental series. Chromatographic conditions are those recommended by Shandon designed for optimal performance. In the example shown, N, for peak a, is 12,499 theoretical plates, 12,764 for peak b and 12,764 for peak c. The maximum quoted theoretical plate value, for ODS-hypersil columns, is 15,000 plates per 25cm (60,000 plates/ metre Shandon Ltd.). The efficiency of the column was therefore approximately 85% of the theoretical value which was deemed sufficient for the present studies.

6.2.4. Standards for HPLC.

Compounds used as standard markers were purchased from

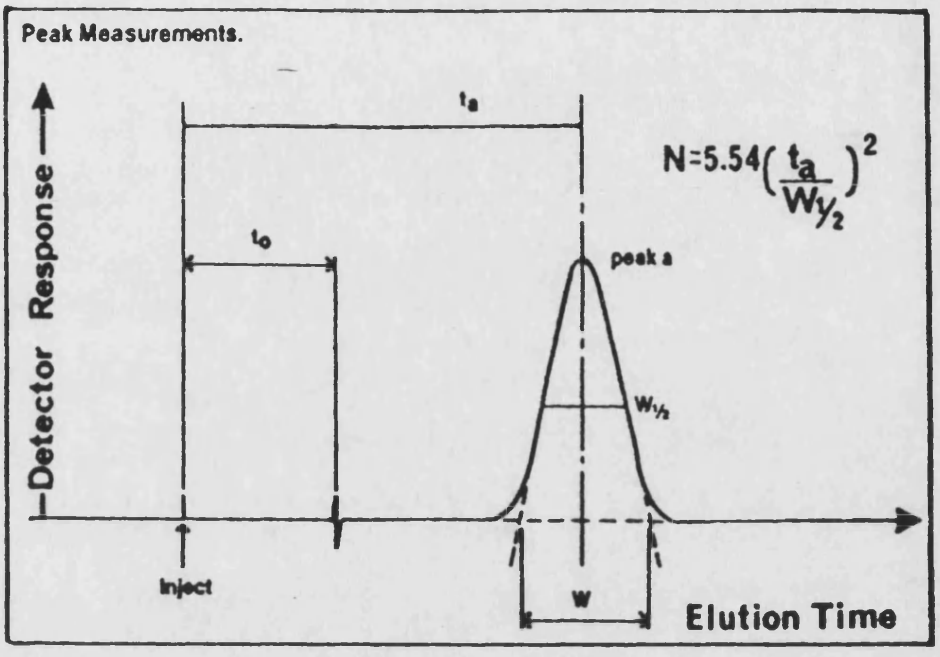


Figure 6.1. The determination of HPLC column efficiency expressed as theoretical plates (N).
t_o = elution time for an unretained solute.
t_a = elution time for solute a.
$W^1/_2$ = peak width at half peak height.
W = peak width at peak base.

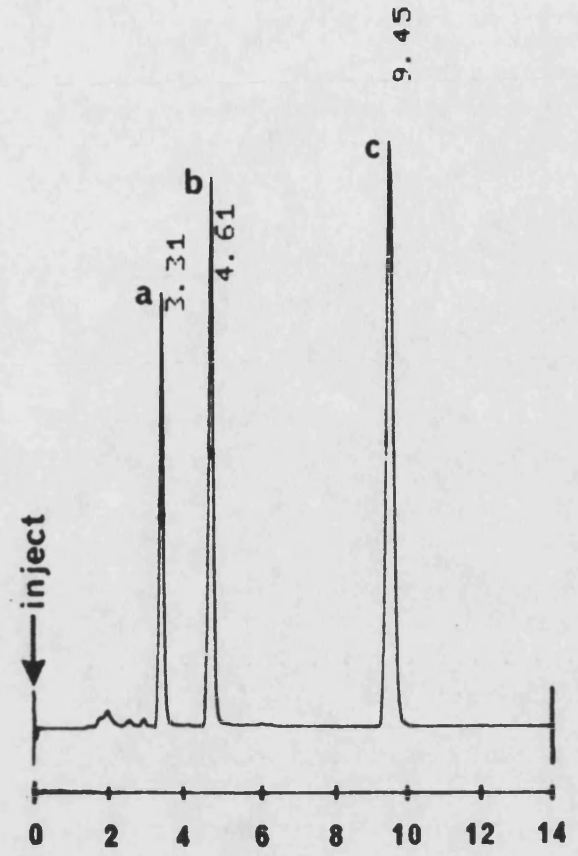


Figure 6.2.

Chromatogram showing separation of the recommended test mixture for ODS-hypersil columns.

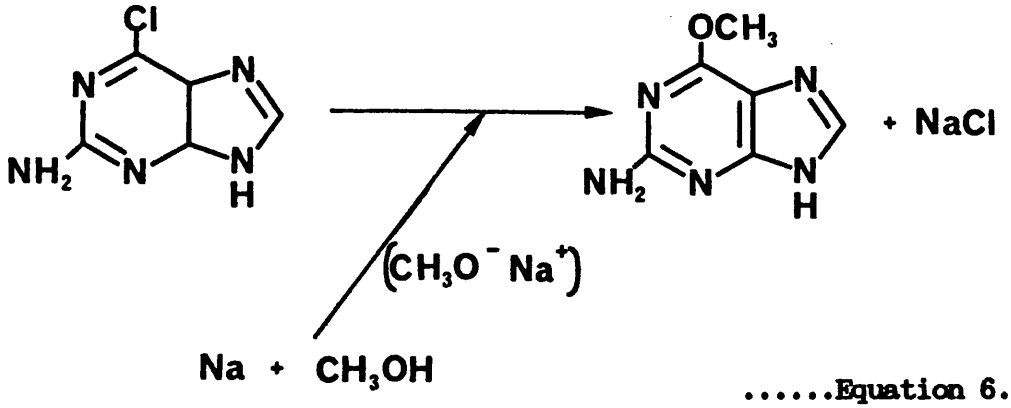
Peak a = Benzamide 3.7mg
Peak b = Acetophenone 0.6mg
Peak c = Benzophenone 0.7mg made to 100ml with mobile phase.
Mobile phase C = methanol:H_2O (70:30)
Flow rate = 1ml min^{-1}. Wavelength = 254 nm.
Chart speed = 0.5 cm min^{-1}. Temperature = ambient.
Apparatus = Spectra-physics (6.2.2.). Loop volume = 20µl.

Aldrich (cytosine, guanine), Sigma (adenine, thymine, 7-methylguanine) and Fluka AG, Switzerland (1-methylguanine, 3-methylguanine, 3-methyladenine). All compounds were dissolved in 0.1M HCl, filtered through a 25 mm x 0.45 µm Millipore filter unit and then diluted with mobile phase to concentrations of 10 $\mu g ml^{-1}$.

The methylated base crucial to these studies, O^6-methyl guanine (O^6MG) is not commercially available and was therefore synthesised at this University. This methylated base was used as a standard marker at a concentration of 5 $\mu g ml^{-1}$.

6.2.4.1. Synthesis of O^6-methylguanine (6-methoxy-2-aminopurine).

Synthesis of this compound was carried out using the method of Balsiger and Montgomery, (1960), which involved the single-step methoxylation of 6-chloroguanine (2-amino-6-chloropurine) according to equation 6.2.



.....Equation 6.2.

The synthesis was performed as follows:

i) Sodium metal (500mg) was dissolved in 20ml of dry methanol in a round-bottomed flask.

ii) 410mg of 2-amino-6-chloropurine HCl (2.4mmoles)(Sigma.) were added and the mixture stirred continuously.

iii) The flask was brought to reflux temperature (80°C) and left for 18 hours attached to a vertical water condenser.

iv) Following reflux the mixture was cooled to room temperature and acidified with 1.2ml (20mmol) of glacial acetic acid. Volatile material was removed under vacuum.

v) The residue was recrystallised from a small quantity of water and dried in a vacuum dessicator after recovery by filtration.

vi) The dried product (O^6MG) (M.Wt = 165.1)was weighed and the yield calculated

2.4mmol of 6-chloroguanine yielded 376mg product i.e.

376/165.1 = 2.28 mmols.

Therefore yield = 2.28/2.4 x 100 = 95%

vii) The structure and purity of the product were verified by NMR spectroscopy.

6.2.4.2. ^1H and ^{13}C NMR spectra of O^6-methylguanine.

The structure of O^6-methylguanine produced by the reaction in 6.2.4.1. was confirmed by NMR spectroscopy. Both reaction compounds were dissolved in DMSO-d_6 (TMS internal standard) and analysed in a JEOL JNM-GX270 FT-NMR spectrometer operating at 270 MHz (^1H) or 67.8 MHz (^{13}C). The ^1H NMR spectra for 6-chloroguanine and the synthesised O^6MG are illustrated in Figure 6.3. For the purposes of this thesis it is not considered necessary to give a detailed account of the underlying theoretical basis of this type of spectroscopy; for this the reader is refered to Williams and Fleming, (1980), and related texts. However, explanation of the spectra presented in Figure 6.3. is facilitated by a brief and concise description of the principles involved in NMR

spectroscopy.

Nuclear Magnetic Resonance (NMR) is observable because certain nuclei e.g. ^{1}H and ^{13}C behave like bar magnets . If a solution of the compound, containing ^{1}H or ^{13}C, is placed in a constant magnetic field the nuclei will either align themselves with the field, in a low-energy orientation, or against the field in a high-energy orientation. At equilibrium there will be a greater proportion of nuclei in the low-energy orientation. Application of an excitory radio-frequency radiation to the solution will cause the nuclei to change their energy state. The difference in energy between the two orientations is given by ΔE, which is proportional to the resonance radiofrequency. The NMR signal is generated by the net absorption of radiofrequency due to the imbalance in distribution of the nuclei between the two energy states. This absorption of a radiofrequency is detected by a receiver and then relayed to a chart recorder.

Nuclei, within a molecule, usually have unique magnetic environments. Each has a slightly different resonance frequency which is characterised by a chemical shift. Using tetramethylsilane ($(CH_3)_4Si$) as the internal reference compound, the chemical shift of a sample proton can be defined in terms of δ

$$\delta = V_S - V_{TMS} \,/\, \text{operating frequency} \times 10^6 \text{ p.p.m.}$$

where V_S = resonance radiofrequency of the sample.
and V_{TMS} = resonance radiofrequency of the internal standard TMS.

The spectrophotometer used for this analysis is equipped with an integration facility in which the spectrum is re-scanned in the mode such that the chart pen rises, on passing a signal in the spectrum, by an amount proportional to the number of protons producing the signal. Knowing that the number of protons of

6-chloroguanine is 4, the relative heights of each step of the integration trace of spectrum 1 (Fig.6.3.), passing from right to left, indicates two, one and one protons at δ values of 6.8, 8.2 and 13 p.p.m. respectively.

Since the only difference, in proton NMR terms, between the starting compound and product is the presence of a methoxyl group, spectrum 2 shouldpossess the same peak and integration patterns as spectrum 1 with the addition of a peak with a relative integration value of 3 i.e. 3 protons. This is indeed the case with the peak corresponding to the methoxyl group positioned at δ value = 4. The peak at 13 p.p.m. was not included in spectrum 2 (Figure.6.3.). It can be concluded, therefore, that a methoxyl group has been substituted at the 6 position of the starting compound by replacing the chlorine atom. Spectrum 2 also shows small peaks to the left of the main peaks at δ values 6.6 and 8.1 p.p.m. These correspond to the presence of a quantity of unreacted starting compound. Measurement of these peaks and comparison with the major peaks at δ values of 6.3 and 7.9 p.p.m. gives an estimation of product purity. When the peaks between 6 and 7 p.p.m. of spectrum 2 are measured, the following is observed:
Starting compound peak height= 27mm. Product peak height = 180mm.
Therefore the purity of the synthesised O^6-methylguanine
is 180/27 x 100 = 85%.

The ^{13}C-NMR spectra of 6-chloropurine and O^6-methylguanine are shown in Figure 6.4. Spectrum 1 expresses peaks corresponding to the aromatic carbon atoms in 6-chloroguanine. Spectrum 2 also contains these peaks but this time also a peak at δ value = 53. This corresponds to the methoxyl group of O^6-methylguanine and is consistent with quoted resonance values for methoxyl groups i.e. δ

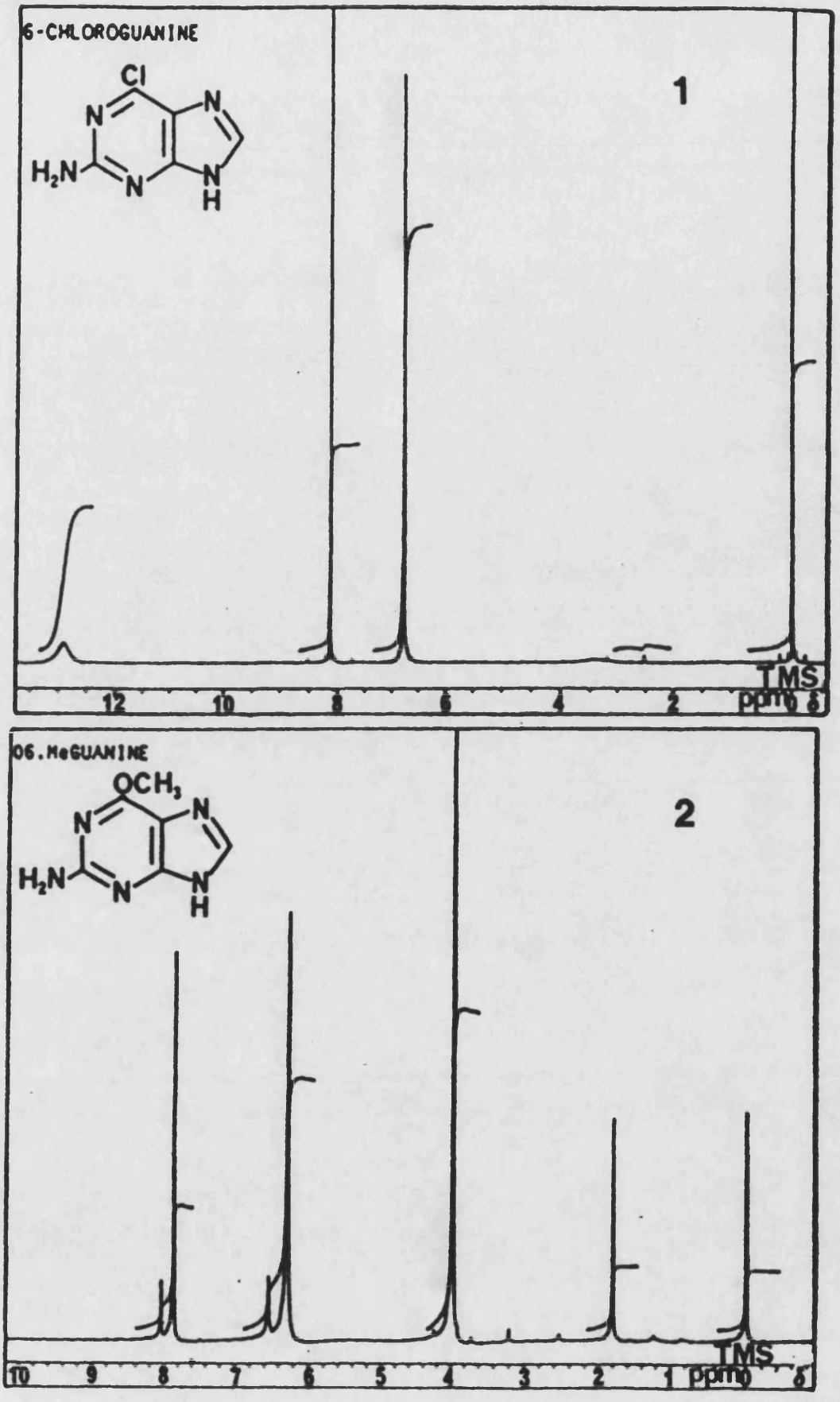


Figure 6.3. Proton NMR spectra of 6-chloroguanine and O^6-methylguanine. Spectrum 2 shows the presence of a methyl group i.e. δ value = 4. (6.2.4.2.).

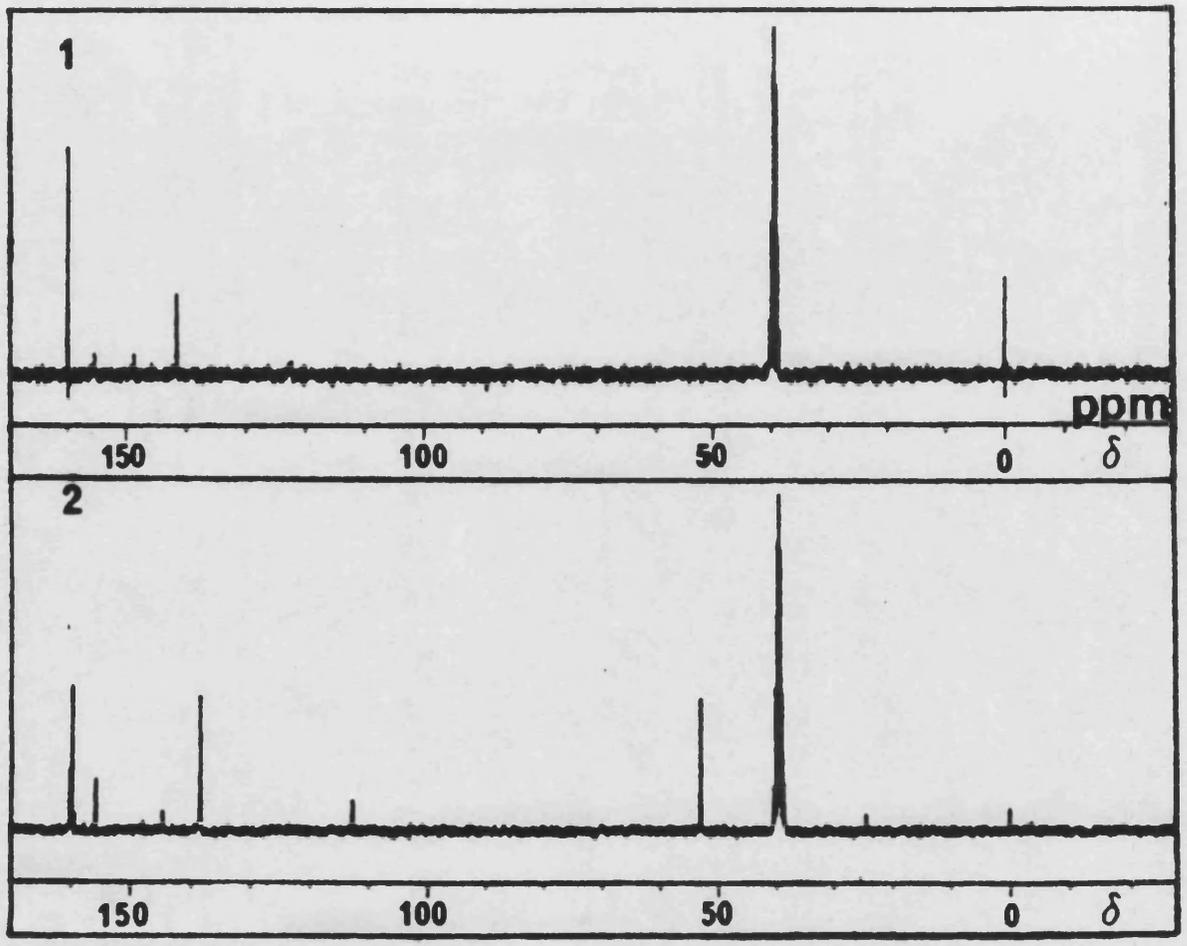


Figure 6.4. ^{13}C-NMR spectra of 6-chloroguanine (1) and the product of equation 6.2. (i.e. the synthesised O^6-methylguanine) (2).

= 45-60ppm (Williams and Fleming, 1980).

These data, in conjunction with the proton-NMR data, are conclusive evidence that the compound produced by the reaction given in 6.2.4.1. is indeed O^6-methylguanine.

6.3. Separation of methylated DNA bases by high-performance liquid chromatography.

6.3.1. Separation of a mixture of 8 DNA bases.

Using the HPLC system outlined in 6.2.1. and 6.2.2. a mixture of 8 DNA bases, (guanine, cytosine, adenine, thymine, 1-methyladenine, 3-methylguanine, 7-methylguanine and O^6-methylguanine) were chromatographically separated. Since the injection loop volume was 20 µl, 10 $\mu g ml^{-1}$ or 1 $\mu g ml^{-1}$ solution injections would load either 200ng or 20ng of base onto the column. Fig.6.5. shows the separation of the above bases using moblie phase A (6.2.1.) at a flow rate of 1 $ml min^{-1}$. Each peak indicates the retention time as calculated by the SP4200 computing integrator. The position of individual bases was determined by preparing a series of eight DNA base solutions, omitting a different base in each, of the eight present in the standard base mixture above, then identifying the absent peak from the standard chromatogram. Table 6.1. shows the mean retention time of each base determined from four replicate chromatographic separations.

The elution order of these bases agrees with the findings of Valencia *et al.*, (1985) who used a reversed phase system, employing an identical mobile phase, to separate 13 nucleic acid bases. The retention times, however, are not comparable since these workers used a 300mm column packed with µBondpak phenyl (C_{18}) with mid-run flow-rate alterations. This system failed to

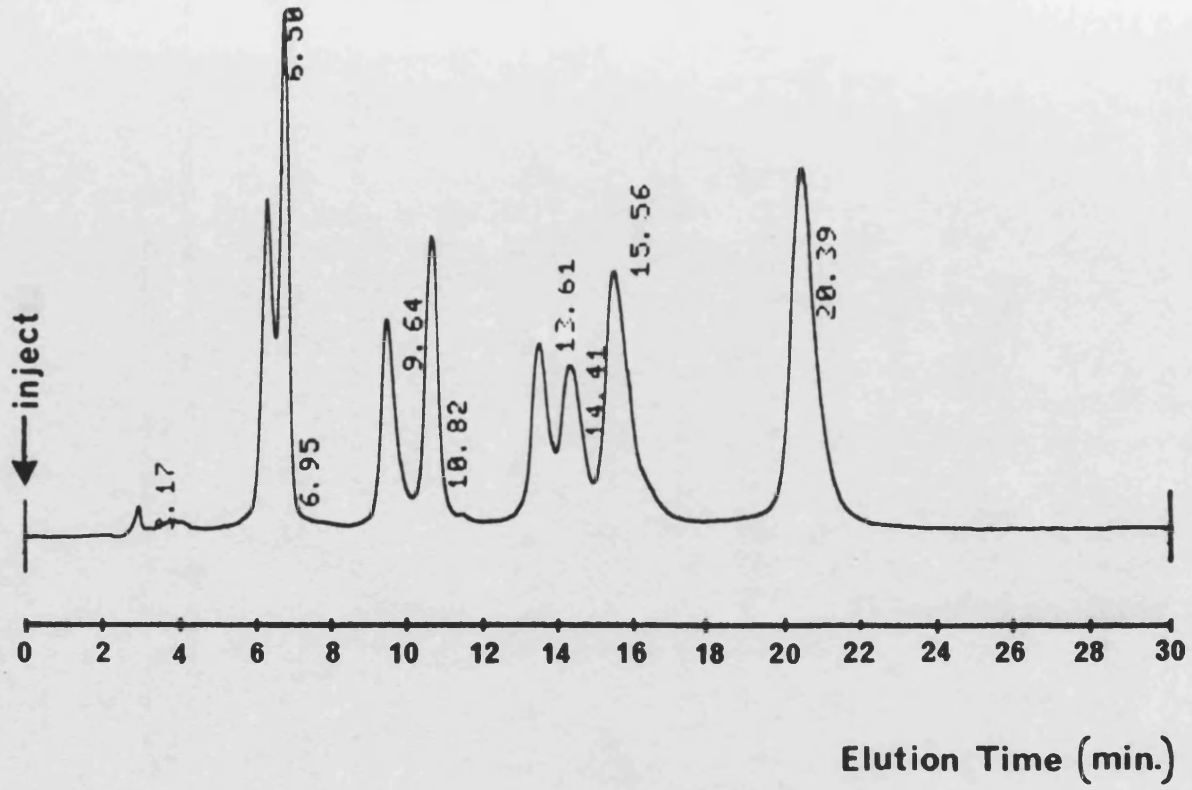


Figure 6.5. Chromatogram showing the separation of 8 DNA bases using an ODS-hypersil column. In the running order, from left to right, the peaks represent cytosine, guanine, thymine, 3-methylguanine, 1-methylguanine, 7-methylguanine, adenine and O^6-methylguanine. Mobile phase A. Flow rate = 1 ml min^{-1}. Wavelength = 254nm. Chart speed = 0.5 cm min^{-1}. Temperature = ambient. Apparatus = Spectra-Physics (6.2.2.). Loop volume = 20 µl.

Table 6.1. The retention times of eight DNA bases separated by reversed-phase HPLC.

Base	Retention Time R_t (mins.)				Average R_t(mins).
Cytosine	6.5	6.58	6.12	6.29	6.37
Guanine	6.95	6.85	7.14	7.14	7.02
Thymine	9.64	9.61	10.18	10.15	9.90
3-methylguanine	10.82	10.81	10.79	10.79	10.80
1-methylguanine	13.61	13.61	13.48	12.84	13.40
7-methyladenine	14.41	14.37	14.68	13.48	14.24
Adenine	15.56	15.59	15.21	15.17	15.38
O^6-methylguanine	20.39	20.39	19.75	19.91	20.11

Column = 250mm x 4.9 mm i.d. ODS-Hypersil (5 µm particle size). Injector loop volume = 20 µl. Temperature = ambient. Mobile phase = A (6.2.1.). Flow rate = 1 ml min^{-1}. Wavelength = 254 nm. Chart speed = 0.5 cm min^{-1}.

separate adenine and 7-methylguanine whilst showing distinct separation of cytosine and guanine. The ODS-hypersil column used in these studies, showed the opposite (Fig.6.5.). It was not possible to resolve 3-methyladenine using this system.

A number of studies have shown adequate separation of bases using strong cation-exchange (SCX) rather than reversed phase columns (Ratko and Pezzuto, 1985; Saffirman *et al.*, 1984; Warren, 1984), but preliminary studies, using a Whatman Partisil 10 SCX column, met with little success. Therefore the reversed-phase system, described above, was used in this study.

6.3.2. Optimisation of O^6-methylguanine detection.

Since O^6-methylguanine is the most important base and had the longest retention time, it was decided to optimise the detection of this base. Decreased retention time, by means of an increased organic modifier i.e. methanol, results in a poor separation of the remaining bases but an improvement in the O^6-methylguanine peak sharpness. The concentration of methanol was increased to 40% (mobile phase B) resulting in a retention time for O^6MG ofapproximately 6 mins (Fig.6.6.) compared to 20 mins using Mobile phase A (Fig.6.5.). Furthermore the sharpness of the O^6-methylguanine peak was increased to such an extent that the machine attenuation was doubled to bring the peak into chart recorder range. The attenuation function of the computing integrator differs from the sensitivity function by producing a visually 'on-scale' peak, whilst integrating the area below the larger 'off-scale' peak Fig.6.6.

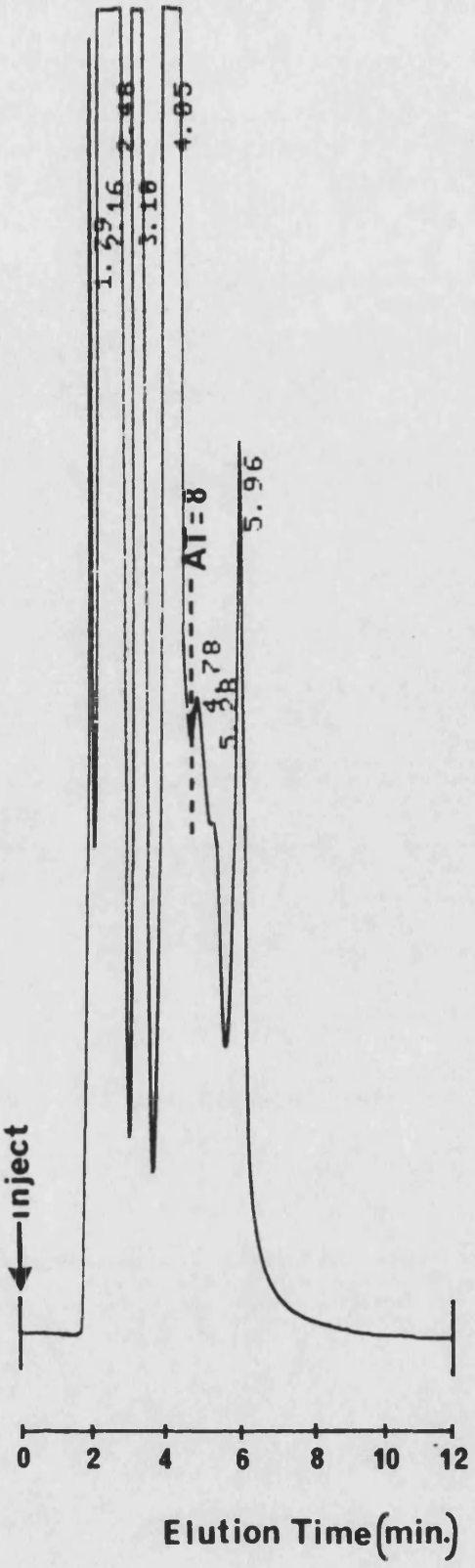


Figure 6.6. Identification of O^6MG (R_t=5.96) in a mixture of 8 DNA bases using N ODS-hypersil column.
Mobile phase B. Flow rate = 1 ml min^{-1}. Wavelength = 254nm.
Chart speed = 0.5 cm min^{-1}. Temperature = ambient.
Apparatus = Spectra-Physics (6.2.2.). Loop volume = 20 µl.

6.3.3. Calibration curve for O^6-methylguanine.

A useful feature of the SP4200 computing integrator is the ability to integrate the area under each chromatogram peak. The areas of fused or riding peaks were calculated by machine estimation of the baseline. Using this feature it was possible to produce an accurate calibration curve of machine integration units plotted against known concentrations of O^6MG and was prepared as shown in Table 6.2. below. this allowed quantification of this base in enzyme assay reaction mixtures Figure 6.7. shows this relationship to be linear over the concentration range tested.

Table 6.2. Preparation of standard solutions for the preparation of an O^6MG calibration curve.

O^6MG stock solution (5 $\mu g ml^{-1}$)(ml).	0.25	0.50	0.75	1.00	1.25
Mobile phase B (ml).	4.75	4.50	4.25	4.00	3.75
Conc^n. of O^6MG ($\mu g ml^{-1}$).	0.25	0.50	0.75	1.00	1.25
O^6MG (ng) loaded onto column (i.e. 20 µl injection).	5	10	15	20	25

6.4. Assay system for the determination of O^6-methylguanine-DNA-methyltransferase (O^6MGMT) activity.

6.4.1. Cell-free extract preparation.

The method of Domoradzki *et al.*, (1984) was used to prepare cell-free extracts of cell lines. This is outlined below:
i) 1.0 - 1.5 x 10^8 cells were suspended in medium, centrifuged at 1000rpm for 5min then washed with buffer containing 50mM Tris-HCl (Sigma), 1mM dithiothreitol (Sigma) and 0.1mM EDTA, pH=7.5 and re-centrifuged at 1000rpm for 5min. ii) The wash was discarded and the pellets used fresh or stored at -80°C until assayed.

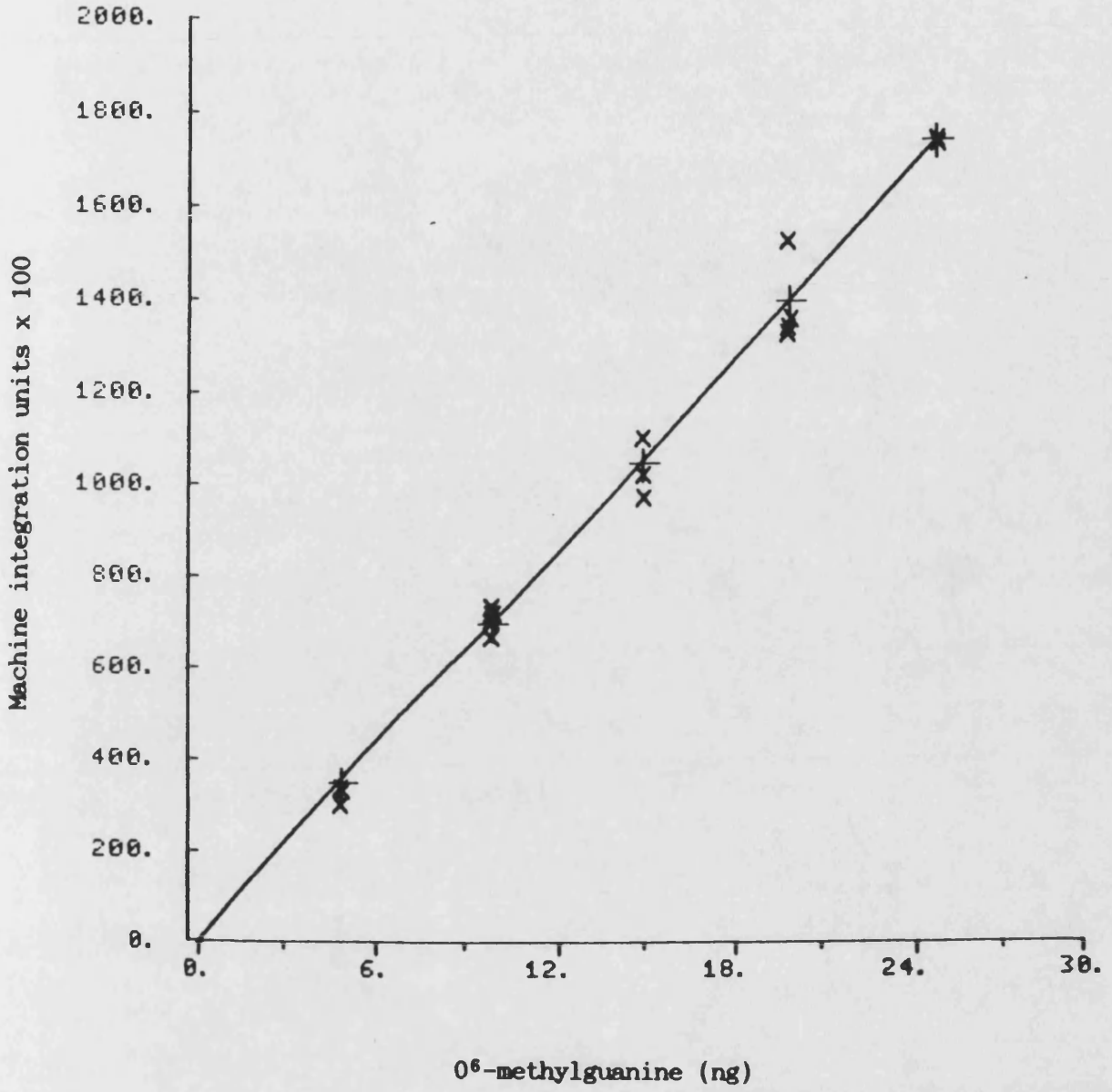


Figure 6.7. Calibration curve of O^6MG against chromatogram peak area. ODS-hypersil column. Mobile phase B. Flow rate = 1 ml min^{-1}. Wavelength = 254. Temperature = ambient. Apparatus = Spectra-Physics. Chart speed 0.5 cm min^{-1}. Loop volume 20 µl. (x) = standard points, (+) = computer fitted line - least squares estimate.

iii) Cell pellets were re-suspended in 1ml of buffer (see (i)) and sonicated in an ultrasonic bath at a frequency of 51 kHz (Lucas Dawe Ultrasonics, Shelton USA.), for two periods of 30 seconds, each separated by a 1 minute interval. The preparation was then centrifuged at 2000rpm for 10 mins to remove cellular debris. The supernatant was removed and designated as the cell extract.
v) Protein concentration in this extract was calculated by the Bradford protein estimation (6.4.4.) and adjusted to 20-30 $mgml^{-1}$ with buffer (see (i)).

6.4.2. Preparation of alkylated DNA substrate.

DNA was alkylated as described by Pegg *et al.*, (1981). Calf thymus DNA was suspended at 5 $mgml^{-1}$ in 80mM sodium phosphate (BDH chemicals), pH=8.0 and incubated with 1mM MNNG for 30 minutes at 37°C. The preparation was dialysed overnight against two changes of phosphate buffer at 4°C.

6.4.3. Enzyme assay conditions.

The reaction was based on the assay used by Boiteux and Laval, (1985) and was comprised of the following:

200 µl cell extract containing between 2 and 5 mg protein (6.4.1.)
200 µl alkylated 5 $mgml^{-1}$ calf thymus DNA suspension i.e. 1mg DNA (6.4.2.)
100 µl incubation buffer (70mM Hepes (Sigma), 1mM EDTA and 1mM dithiothreitol, pH=7.6).

This mixture was incubated at 37°C for 0, 30 or 60 minutes in an Eppendorf microfuge tube (Sterilin) and the reaction stopped by rapidly cooling to 0°C on ice. DNA hydrolysis was achieved by

addition of 20 µl 1M HCl and then heating the tubes at 80°C for 30 minutes. The samples were neutralised by addition of 20 µl 1M NaOH and the precipitate removed by centrifugation at 12,000g for 5 min. in a MSE Microcentaur microfuge (2.1.) The supernatant was filtered through a 25mm Swinnex unit fitted with a 0.2 µm Sartorius membrane (2.5.1.) and loaded onto the HPLC system for base quantification (6.5.).

6.4.4. Protein estimation.

Protein estimation was carried out by the method of Bradford, (1976) using a protein dye binding assay system:

Standard protein reagent - 1 $mgml^{-1}$ Bovine Serum Albumin (BSA) (Aldrich) in distilled water.

Protein reagent - 100mg of Coomassie Brilliant Blue G-250 (BDH) was dissolved in 50ml of 95% ethanol. 100ml of 85% phosphoric acid w/v (FSH) was added and the solution made to 1 litre with distilled water i.e. 0.01% v/v Coomassie Blue, 4.75% w/v EtOH and 8.5% phosphoric acid.

Standard protein curve - Duplicate standard curves were prepared as shown in Table 6.3. below:

Table 6.3. Preparation of standard solutions for the construction of a protein calibration curve.

Standard solution (µl)	0	10	20	40	60	80	100
Water (µl)	100	90	80	60	40	20	0
Protein (total µg)	0	10	20	40	60	80	100

To each tube 5ml of protein reagent were added, vortex

mixed and and then left for 5 minutes at room temperature. The optical density at 595nm was read and a standard curve constructed. The spectrophotometer was set to zero by using a blank containing water. Fig.6.8. shows a typical protein standard curve of optical density at 595nm plotted against µg protein (BSA) and is seen to be linear with increasing dose over the range 10 to 80 µg protein, above which the relationship becomes non-linear.

Protein estimation of cell-free extracts used 100µl plus 5ml protein reagent. If the optical density was greater than the range of the standard curve the sample was diluted with buffer (6.4.1.(i)) and re-estimated. A standard curve was constructed before each protein estimation since the protein reagent is known to deteriorate with age (Bradford, 1976).

6.4.5. Treatment of results.

Cell-free extract mediated de-methylation of the alkylated DNA substrate was assessed by comparison of the total amount of O^6MG, at incubation time zero, with the total amount of O^6MG after 30 and/or 60 minutes incubation at 37°C (6.4.3.). An O^6MG calibration curve was constructed immediately before sample analysis and the amount, in nanogrammes, was calculated then converted to pmoles by multiplying by a factor of 6 (M.Wt. of O^6MG = 165.1, hence 1ng = 6pmoles).

Results are expressed as follows:

pmoles O^6MG de-methylated/mg protein =

pmoles O^6MG de-methylated/mg protein at t=zero

- pmoles O^6MG de-methylated/mg protein at t=30 or t=60

.....Equation 6.3.

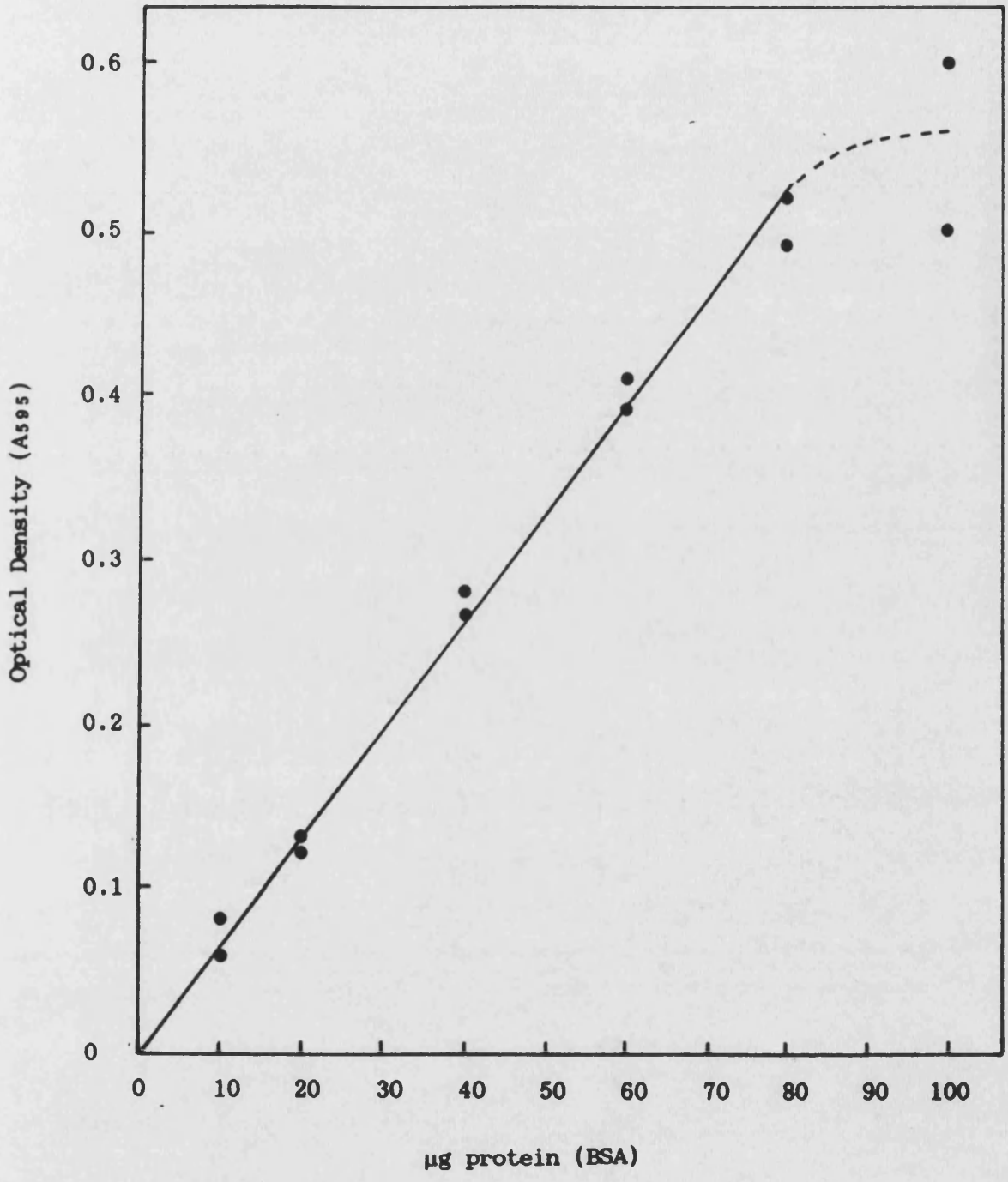


Figure 6.8. Protein standard curve estimated by the method of Bradford, (1976). Optical density at 595nm is plotted against µg BSA standard.

6.5. De-methylation of O^6MG in an exogenous alkylated DNA substrate.

6.5.1. Assessment of the ability of C3H $10T^1/_2$ cells to de-methylate O^6MG of an exogenous alkylated DNA substrate.

C3H $10T^1/_2$ cells have been shown to possess the ability to de-methylate O^6MG adducts contained within MNNG-treated DNA (Grisham and Smith, 1984; Smith *et al.*, 1981). This cell line was therefore used as a positive control for the validation of the assay system. C3H $10T^1/_2$ cells are a fibroblast line derived from the embryonic ventral prostrate glands of inbred C3H mice (Reznikoff *et al.*, 1973). They have a plating efficiency of 12 to 20%, an average generation time of 15.5 hours and are routinely grown in $75cm^2$ T/C flasks (2.1.). In previous studies Eagles Basal medium (BME) was used for the culture of this cell line (Greenberg *et al.*, 1978; Reznikoff *et al.*, 1973; Smith *et al.*, 1981), but the use of Hams F10 containing 10% heat-inactivated FCS supported adequate cellular growth, with a doubling time of 15 hours.

The de-methylation ability of this cell line was assessed as follows:

Two days prior to the experiment twelve $75cm^2$ flasks, each containing 15ml medium, were inoculated with $1x10^6$ C3H $10T^1/_2$ cells, gassed with 5% CO_2 in air and incubated at 37.5°C. After 18 hours growth the cells were dispersed with trypsin (2.3.3.), a cell extract prepared as described in 6.4.1. and the protein content estimated (6.4.4.) and adjusted to 20-25 $mgml^{-1}$. As an approximation, for experimental guidance, $1x10^8$ cells were equivalent to 20mg protein.

Incubation of each cell extract with alkylated DNA substrate, and its subsequent hydrolysis, was performed as outlined in 6.4.3.. The supernatants of extracts incubated at t=0,

30 and 60 minutes were analysed for O^6MG content by the HPLC system outlined in 6.3.2. Fig.6.9. shows a chromatogram series, from a single experiment, of total O^6MG content after each incubation time. Table 6.4. presents the results obtained from four independent experiments. The ability of C3H $10T^1/_2$ cell extracts to de-methylate O^6MG from alkylated DNA is expressed as pmoles O^6MG removed per mg cell extract protein. After 30 and 60 minutes incubation an average of 8.3 and 23.3 pmoles per mg protein, were de-methylated by the extracts. Smith *et al.*, (1981) reported the capacity of this cell line to de-methylate O^6MG and so it was decided that the C3H $10T^1/_2$ cell line could act as a satisfactory positive control for these studies.

6.5.2. Assessment of the ability of CHO-K1, CHO-K1R, V79-379A and V79-379AR cells to de-methylate O^6MG.

The experimental protocol for the above cell lines was identical to that used for the C3H $10T^1/_2$ cell line as described in 6.5.1. The de-methylation of O^6MG from alkylated DNA by each cell line is shown in Table 6.5. From these results it can be seen that there is no significant de-methylation of O^6MG by cell extracts of any of these cell lines. The control consisted of 5mg BSA substituted for the cell extract. It seems probable, therefore that neither CHO-K1 nor V79-379A cells possess any O^6MG de-methylating ability i.e. no methyltransferase enzyme, even at a constituitive level. The MNNG-resistant cells CHO-K1R and V79-379AR, which have been shown to have a lower MNNG-induced mutation frequency than their respective parental cells (5.6.1.), also exhibited the lack of ability to de-methylate the alkylated substrate. The partial de-methylating ability of each cell line

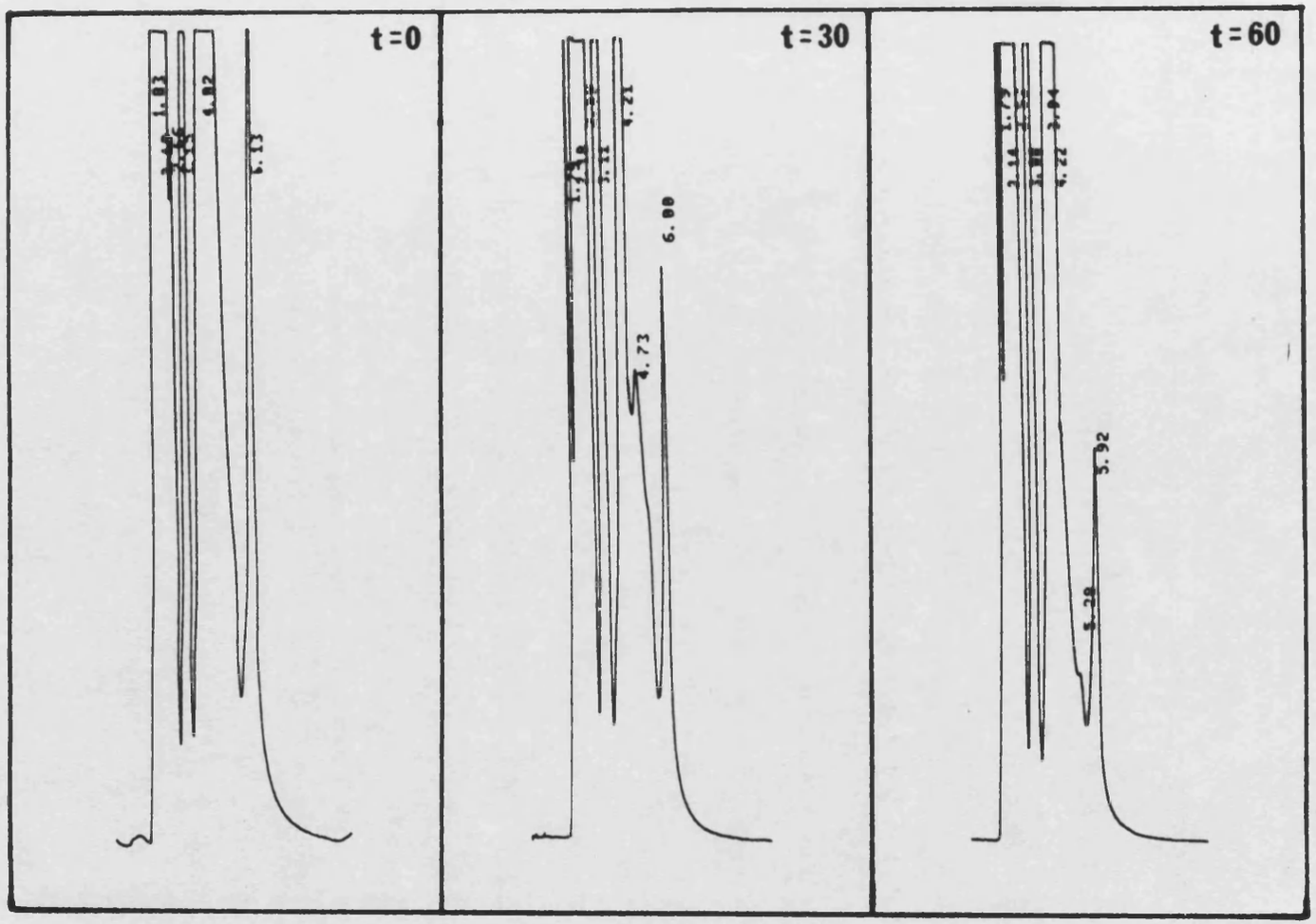


Figure 6.9. Chromatogram series illustrating the progressive disappearance of the O^6MG peak as a function of incubation time with a cell-free extract of C3H $10T^1/_2$ cells.
ODS-hypersil column. Mobile phase B. Flow rate = 1 ml min^{-1}. Wavelength = 254nm. Chart speed = 0.5 cm min^{-1}. Temperature = ambient. Apparatus = Spectra-Physics (6.2.2.). Loop volume = 20µl.

Table 6.4. De-methylation of O^6MG in alkylated DNA by cell-free extracts of the C3H $10T^1/_2$ cell line.

Cell line	Incubation time (mins).	Expt N°.	O^6MG peak area (machine units).	ng	pmoles	Cell extract total mg/ incubation.	pmoles O^6MG removed/mg protein*.	Mean±S.E.
C3H $10T^1/_2$	0	1	191565	27.4	164	4.8	0	
		2	195736	28.0	168	5.1	0	0
		3	203712	29.2	175	4.9	0	
		4	216346	30.9	185	4.8	0	
	30	1	153626	21.9	131	4.8	6.9	
		2	154712	22.1	133	5.1	6.8	8.3±0.86
		3	153630	21.9	131	4.9	8.9	
		4	157220	22.5	135	4.8	10.4	
	60	1	68360	9.8	59	4.8	21.9	
		2	72854	10.4	62	5.1	20.8	23.3±1.19
		3	63219	9.1	55	4.9	24.5	
		4	70184	10.0	60	4.8	26.0	

* Calculated by Equation 6.3.

Table 6.5. De-methylation of O^6MG in alkylated DNA by cell-free extract of CHO-K1 and V79-379A cells and their respective MNNG resistant clones CHO-K1R and V79-379AR.

Cell line	Incubation time (mins).	Expt. N°.	O^6MG peak area (machine units).	ng	pmoles	Cell extract total mg/ incubation.	pmoles O^6MG removed/mg protein*.	Mean.
CHO-K1	0	1	203242	29.2	174	5.0	0	0
		2	211544	30.5	182	4.9	0	
	60	1	200489	28.8	172	5.0	0.4	0.4
		2	219951	31.8	190	4.9	0	
CHO-K1R	0	1	203815	29.2	175	4.8	0	
		2	213861	30.4	183	4.7	0	
	60	1	194700	27.9	167	4.8	1.7	
		2	193771	17.7	166	4.7	3.6	2.7
V79-379A	0	1	192832	27.6	165	4.2	0	
		2	216841	31.0	186	4.6	0	
	60	1	191643	27.4	165	4.2	0	
		2	187241	26.8	161	4.6	5.4	5.4
V79-379AR	0	1	197467	28.3	170	4.2	0	
		2	198447	28.4	170	5.0	0	
	60	1	193241	27.7	166	4.2	0.95	
		2	197389	28.3	170	5.0	0	0.95
Control (5mg BSA).	0	1	204952	29.3	176	5.0	0	
		2	214831	30.7	184	5.0	0	
	60	1	191643	27.4	164	5.0	2.4	
		2	197210	28.2	169	5.0	3.0	2.7

*Calculated by Equation 6.3.

was attributed to spontaneous substrate de-methylation since a control, which substituted BSA for the cell-free extract, also showed a small degree of de-methylating ability, after 60 minutes incubation i.e. 2.7 pmoles de-methylated/ mg protein for CHO-K1 cells and 3.7 pmoles de-methylated/ mg protein for V79-379A cells (Table 6.5.).

6.5.3. The effect of adaptive pre-treatment on the ability of CHO-K1, CHO-K1R, V79-379A and V79-379AR cells to de-methylate O^6MG.

Two days prior to the start of the experiment, twelve 75cm^2 T/C flasks, each containing 15ml Hams F10 + 5% FCS were inoculated with 1x10^6 cells. To each flask 0.01 $\mu g ml^{-1}$ MNNG was added (t=-48). The flasks were gassed with 5% CO_2 in air and incubated at 37.5°C. After 6 hours (t=-42), and every six hours until t=0, the medium in each flask was replaced with fresh, prewarmed Hams F10 + 5% FCS containing 0.01 $\mu g ml^{-1}$ MNNG. At t=0 all cells were harvested and assayed for their ability to de-methylate O^6MG as outlined in 6.5.2. The evidence of 3.5.2. and the results of the above experiments, presented in Tables 6.6. and 6.7., strongly suggest the absence or inactivity of the O^6MGMT enzyme in the cell lines studied since the de-methylation observed was not greater than that found for the protein control.

Table 6.6. De-methylation of O^6MG in alkylated DNA by cell-free extracts of CHO-K1 and CHO-K1R cells following adaptive pre-treatment.

Cell line	Incubation time (mins).	Expt. N°.	O^6MG peak area (machine units).	ng	pmoles	Cell extract total mg/ incubation.	pmoles O^6MG removed/mg protein*.	Mean.
CHO-K1	0	1	203242	29.2	174	5.0	0	0
		2	211544	30.5	182	4.9	0	
	60	1	200489	28.8	172	5.0	0.4	0.4
		2	219951	31.8	190	4.9	0	
CHO-K1	0	1	213338	30.5	183	5.2	0	
+ ADA		2	197015	28.2	169	5.1	0	
	60	1	183150	26.2	156	5.2	5.2	
		2	190565	27.3	164	5.1	0.98	3.1
CHO-K1R	0	1	203815	29.2	175	4.8	0	
		2	213861	30.4	183	4.7	0	
	60	1	194700	27.9	167	4.8	1.7	
		2	193771	17.7	166	4.7	3.6	2.7
CHO-K1R	0	1	197562	28.3	170	4.9	0	
+ ADA		2	213861	30.6	183	4.7	0	
	60	1	192654	27.6	165	4.9	1.0	
		2	193718	27.7	166	4.7	3.6	2.3
Control	0	1	204952	29.3	176	5.0	0	
(5mg BSA).		2	214831	30.7	184	5.0	0	
	60	1	191643	27.4	164	5.0	2.4	
		2	197210	28.2	169	5.0	3.0	2.7

*Calculated by equation 6.3. ADA = adaptive pre-treatment (Protocol 2).

Table 6.7. De-methylation of O^6MG in alkylated DNA by cell-free extracts of V79-379A and V79-379AR cells following adaptive pre-treatment.

Cell line	Incubation time (mins).	Expt. N°.	O^6MG peak area (machine units).	ng	pmoles	Cell extract total mg/ incubation.	pmoles O^6MG removed/mg protein*.	Mean.
V79-379A	0	1	192832	27.6	165	4.2	0	
		2	216841	31.0	186	4.6	0	0
	60	1	191643	27.4	165	4.2	0	
		2	187241	26.8	161	4.6	5.4	5.4
V79-379A	0	1	206133	29.5	177	4.3	0	
+ ADA		2	198324	28.4	170	5.1	0	
	60	1	193128	27.6	166	4.3	2.5	
		2	197770	28.3	170	5.1	0	2.5
V79-379AR	0	1	197467	28.3	170	4.2	0	
		2	198447	28.4	170	5.0	0	
	60	1	193241	27.7	166	4.2	0.95	
		2	197389	28.3	170	5.0	0	0.95
V79-379AR	0	1	218734	31.3	188	5.1	0	
+ ADA		2	204562	28.3	169	5.0	0	
	60	1	198234	28.4	170	5.1	3.5	
		2	196685	28.1	169	5.0	0	3.5
Control	0	1	204952	29.3	176	5.0	0	
(5mg BSA)		2	214831	30.7	184	5.0	0	
	60	1	191643	27.4	164	5.0	2.4	
		2	197210	28.2	169	5.0	3.0	2.7

*Calculated by Equation 6.3. ADA = adaptive pre-treatment (Protocol 2).

6.6. Conclusions.

The following conclusions can be drawn from the results presented in this chapter.

1) Methylated and normal DNA bases can be separated and identified by HPLC using a reversed-phase ODS-hypersil column (6.3.).
2) The total content of O^6MG in an acid hydrolysed DNA sample can be calculated using a calibration curve of peak area plotted against nanogramme quantities of O^6MG standard. The HPLC technique is capable of quantitative determination of O^6MG over the range 5-20 ng.
3) Mouse fibroblast cells C3H $10T^1/_2$ are able to de-methylate O^6MG contained within an exogenous methylated DNA substrate. Cell-free extracts of C3H $10T^1/_2$ removed 8.3 pmoles O^6MG per mg protein after 30 minutes incubation and 23.3 pmoles per mg protein after 60 minutes (Table 6.4.).
4) Cell-free extracts of CHO-K1, CHO-K1R, V79-379A and V79-379AR cells did not change the amount of O^6MG in methylated DNA after 60 minute incubation. The partial de-methylating ability of each cell line was attributed to spontaneous substrate de-methylation since a control, which substituted BSA for the cell-free extract, also showed a small degree of de-methylation ability i.e. 2.7 pmoles de-methylated/ mg protein/ hour (Table 6.5.)
5) Adaptive pre-treatment with non-toxic doses of MNNG did not alter the de-methylation ability of the four hamster cell lines studied (Tables 6.6. and 6.7.).
6) It can be concluded, therefore that CHO-K1, V79-379A and the derived MNNG-resistant clones do not possess an active O^6MGMT enzyme, even at a low, constitutive level.

These conclusions will be discussed in Chapter 8.

CHAPTER 7. THE EFFECT OF ADAPTIVE PRE-TREATMENT ON THE MNNG-INDUCED SISTER CHROMATID EXCHANGE FREQUENCIES IN CHINESE HAMSTER CELLS.

7.1. Introduction.

Sister chromatid exchanges (SCE), originally demonstrated by Taylor, (1958), reflect the symmetrical, reciprocal interchange of DNA molecules between sister chromatids at homologous loci. The introduction of high-resolution bromodeoxyuridine (BrdU) dye techniques has greatly simplified the visualisation of SCE in metaphase chromosome preparations (Latt, 1975; Perry and Wolff, 1974). These techniques have revealed that SCE occurs at the time of DNA replication and involves an exchange of DNA duplexes (Evans, 1977). DNA damaging agents have been shown to greatly increase the frequency of SCE over the normally low, spontaneous levels evident in untreated cells, and consequently are considered to be a useful indicator of the mutagenicity of these agents (Evans, 1977). Although little is known about the biochemistry of the formation of SCE, it has been suggested that they occur as a result of a cellular DNA repair response to DNA lesions, and are probably some kind of recombination process (Latt *et al.*, 1975; Wolff *et al.*, 1975). Since several classes of agent are capable of inducing SCE, the lesions responsible are probably of many different types (Evans, 1977; Heflich *et al.*, 1982; Morris *et al.*, 1984). Recent reports indicate a correlation between the induction of SCE and mutational events, although no universal conclusions have been drawn about this relationship since each test agent has responded differently, in terms of a mutation to SCE ratio (Carrano *et al.*, 1978; Heflich *et al.*, 1982; Morris *et al.*, 1984;

Natarajan *et al.*, 1984).

Samson and Schwartz, (1980) first reported a decrease in MNNG-induced SCE frequencies following MNNG or MNU adaptive pre-treatment. SCE were reduced in both CHO and in the SV-40 transformed human cell line GM637. Similar results were found by Kaina, (1983b) who reported the protective effect of MNNG pre-treatment on the induced SCE frequency of V79-C10 cells, after subsequent MNNG exposure. HeLa S3, V79 and CHO cells transfected with an plasmid containing an active *E.coli* O^6MGMT gene, show lower induced SCE frequencies than their respective, non-transfected parental line, suggesting that one of the lesions responsible for SCE is O^6MG (Bignami *et al.*, 1987; Ishizaki *et al.*, 1986; White *et al.*, 1986). Induction of this enzyme, under adaptive conditions, may explain the reduction of SCE following pre-treatment. Since CHO and V79 have been shown to lack the O^6MGMT enzyme (Foote *et al.*, 1983; Foote and Mitra, 1984; Chapter 6.5.) it would seem unlikely that any decrease of induced SCE frequency, following adaptive pre-treatment, can be attributed to induction of this enzyme.

The following chapter outlines the techniques involved in SCE visualisation and reports the MNNG-induced SCE of CHO-K1 and V79-379A cells either with or without adaptive pre-treatment. Resistant lines CHO-K1R and V79-379AR were also analysed to establish whether the process giving rise to cytotoxic resistance had any influence on the MNNG-induced SCE process. The sensitive cell clones, CHO-K1S and V79-379AS, were not assessed for MNNG induced SCE because they exhibited such a drastic increase in population doubling time, following adaptive pre-treatment (5.5.2.), that incubation of cells with BrdU, for two rounds of

DNA replicaton, would have unreasonably lengthened the experimental protocol.

7.2. Materials.

Materials used in the assay of SCE, not previously listed in chapter 2, are shown below.

1) 5-bromodeoxyuridine (BrdU) Sigma. M.Wt = 307.4. The solid and 5mM (1.54 $mgml^{-1}$) stock solution in PBS(A) were stored, protected from light, at -20°C.
2) Hoechst 33258 (2-(-2-(4-hydroxyphenyl)-6-benzimidazolyl)-6 -(1-methyl-4-piperazyl)-benzamidazole 3HCl), Hoechst GMbH, Frankfurt, W.Germany. Solid and stock solution in DDH_2O (500 μgml^{-1}) were stored at 4°C protected from light.
3) Polyurethane cement, Woolworth plc, Bath.
4) Broad-spectrum UV-light source - General Electric 15-W blacklight (F15T8 BLB).
5) Sorensen's buffer - 6.7mM potassium dihydrogen phosphate and 6.7mM disodium hydrogen phosphate, pH=6.8.
6) 2 x SSC - saline sodium citrate (0.3M sodium chloride, 0.03M tri-sodium citrate (Sigma), pH=7.0).
7) Glacial acetic acid - Fisons (FSH) Ltd.
8) Microscope slides - 76 x 26mm, 1mm thick, pre-washed microscope slides (KTH 370) (Chance Proper Ltd., Smethwick, England).
9) Colchicine - mitotic spindle inhibitor, (BDH chemicals Ltd), was stored as a dry powder at room temperature. Stock solution of 0.4 $mgml^{-1}$ (1mM) in DDH_2O was stored frozen at -20°C.

7.3. Detection of Sister Chromatid Exchanges (SCE).

SCE detection requires the differential staining of sister chromatids. This is usually achieved by incorporation of the thymidine analogue 5-bromodeoxyuridine (BrdU) into the chromatids

during replication. SCE analysis takes advantage of the semi-conservative nature of DNA replication and if cells are exposed to BrdU for two cell cycles, metaphase chromosomes come to possess one chromatid unifilarly substituted with BrdU and its sister, bifilarly substituted. The chromatids can be differentiated by treatment with the dye Hoechst 33258, which fluoresces more strongly when bound to unifilarly substituted chromatids than when bound to bifilarly substituted chromatids (Latt, 1975). Since the Hoechst dye is highly photo-instable a photographic record must be made of the fluorescing metaphase within one minute of preparation. A more permanent differentiation procedure was devised by Perry and Wolff, (1974) who combined Hoechst dye incorporation with Giemsa staining (Fluorescence plus Giemsa or FPG technique), which allows examination of sister chromatids by conventional light microscopy.

7.3.1. Cell Treatment Protocol.

This protocol was designed such that the cells were exposed to BrdU for two rounds of replication to ensure that virtually all metaphase cells examined were at the second mitosis following BrdU addition.

Cultures were started by inoculating $4x10^5$ cells into $25cm^2$ T/C flasks each containing 5ml Hams F10 + 5% FCS lacking thymidine (2.3.5.), gassed with 5% CO_2 in air and incubated at 37.5°C. (Thymidine present in the media may compete with BrdU for incorporation into DNA during its synthesis, resulting in a lower SCE rate (Morgan and Crossen, 1977)). After 20 hours incubation the culture media was discarded and replaced with 5ml fresh Hams F10 + 5% FCS lacking thymidine to which 10 μM BrdU had been added.

Test or control treatments were also introduced at this stage. Following 30 hours incubation, 5 μM colchicine was added and two hours later the cells were harvested. Each culture flask was washed with 2ml of trypsin solution (2.3.3.) and then incubated with 3 ml trypsin at 37°C for 5 minutes. The resulting cell suspensions were tipped into plastic T/C serum tubes (2.1.) and 5ml of warm DDH_2O added to each. Thorough mixing of the tubes was followed by a 15 minute incubation in a water bath set at 37.5°C. The cells, swollen by the hypotonic treatment, were centrifuged in a bench centrifuge for 5 minutes at 1000 rpm, and then resuspended in about 0.2ml supernatant. 5ml of fresh fixative (3:1, methanol:glacial acetic acid. Prepared and used within 1 hour), was rapidly added. After centrifugation, the fixative was discarded with a Pasteur pipette, care being taken not to disturb the cell pellet, and replaced with 5ml fresh fixative. After thorough mixing, the tube was re-centrifuged, the fixative discarded and the cell pellet re-suspended in approximately 1ml of fresh fixative. Tubes were left at -20°C for 1 hour before metaphase preparation.

A metaphase chromosome preparation was made by running 3-5 drops of the cold cell suspension down a clean microscope slide held at 45°. The slide was allowed to air-dry in a vertical position and then checked for adequate metaphase density under low-power phase optics. If the density of metaphase cells was too low additional drops of the suspension were added and again left to air-dry. If the metaphase spreads were not satisfactory i.e. did not show separate, well-defined chromosomes in one plane of focus, the cells were subjected to further changes of fixative and re-spread. If this was not satisfactory the spreads were discarded

and the experiment repeated.

7.3.2. Fluorescence plus Giemsa Staining (FPG).

The FPG method of Perry and Wolff, (1974) was used in these studies. Freshly made slides tend to show G-banding rather than the characteristic 'harlequin' staining of SCE preparations. Therefore metaphase spreads were 'aged' for at least 1 week, in a dust-free environment at room temperature. After ageing, slides were stained in Hoechst 33258 0.5 $\mu g ml^{-1}$ (1:1000 dilution of stock with Sorensen's buffer pH=6.8), for 15 minutes. After rinsing with tap water and blotting dry, the preparations were mounted with Sorensen's buffer, a coverslip placed over the slide and sealed with polyurethane cement. The slides were then exposed to broad-spectrum UV-light for 2 hours. This allowed a photochemical reaction to occur whereby chromatids come to fluoresce differentially. Careful removal of the coverslips i.e. lifted straight off rather than slid off, was followed by immersion in 2 x SSC at 60°C and incubated for 1 hour, before finally staining the slides in 5% Giemsa in Sorensen's buffer pH=6.8 for 30 minutes. Slides were then rinsed in DDH_2O and air-dried. Microscope evaluation of SCE was now possible.

7.3.3. Scoring of SCE.

Following FPG staining all slides were coded with a diamond tipped glass marker and scored randomly i.e. 'blind'. Each slide was scanned methodically in breadthwise sweeps under low power (x256) and every apparently unbroken metaphase cell examined under high-power (x1600) oil immersion, bright-field microscopy. Spreads that were completely harlequin stained and which contained between

19 and 22 centromeres were analysed for SCE. A SCE was only counted when it was clearly evident in both chromatids at the same point. Care was taken not to count one SCE where there were two exchange points. Each exchange point, rather than the area between the exchange point, should be scored as an SCE; otherwise the total number of SCE will be under estimated. The chromosome illustrated in Plate 7.1. below shows 3 SCE, as indicated by the arrows.

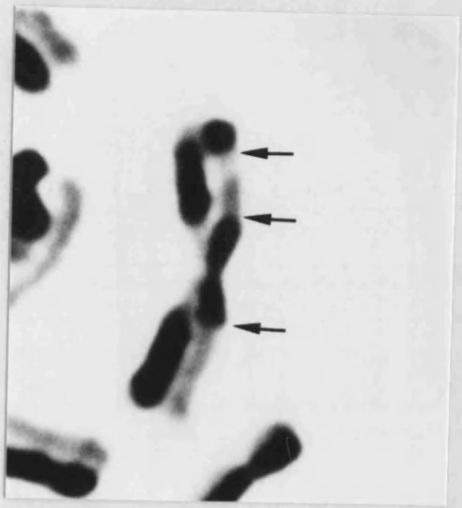


Plate 7.1. A single chromosome from a metaphase preparation of a CHO-K1 cell exhibiting three sister chromatid exchanges (arrowed).

7.3.4. Treatment of results.

Conventionally, the frequency of SCE is expressed as 'SCE per cell' (Carrano *et al.*, 1978; Natarajan *et al.*, 1984; Schwartz, 1986; Thompson *et al.*, 1982) or as 'SCE per chromosome' (Bodell, 1986; Morris *et al.*, 1984; Samson and Linn, 1987). Both parameters have been reported but the latter is probably the most meaningful since 'SCE per cell' is influenced by the variation in chromosome

number, common to many cell lines, and by loss of chromosomes during preparation. In this chapter data are presented both as 'SCE per chromosome' and 'SCE per cell'.

7.4. Dose-response relationship of MNNG-induced SCE.

The construction of an SCE/mutagen dose-response curves served two useful purposes; a) to create a 'standard MNNG-induced SCE curve' for comparison with curves produced by cells subjected to adaptive pre-treatment and b) to evaluate the efficiency of the technique by comparison to SCE yield obtained by other workers.

7.4.1. MNNG-induced SCE in CHO-K1 and V79-379A cells.

The details of cell treatment, staining and scoring of SCE are outlined in 7.3. Duplicate flasks were set up for both control (vehicle) and mutagen treated cells. A range of 0.1 - 0.3 $\mu g ml^{-1}$ MNNG was used and for each dose a total of 19-21 harlequin-stained metaphase cells were scored for SCE. The data obtained from three independent experiments is presented in Tables 7.1. and 7.2. for CHO-K1 and V79-379A cells respectively, and are represented graphically in Figs. 7.1. and 7.2. plotted as SCE per chromosome against MNNG concentration. The S.E. in these, and subsequent Tables in this chapter, were calculated using the individual data points, thus taking sample size into account. In Plate 7.2. a cell from the vehicle control culture and a cell exposed to 0.1 $\mu g ml^{-1}$ MNNG are shown as examples of SCE, as visualised under high-power oil-immersion microscopy.

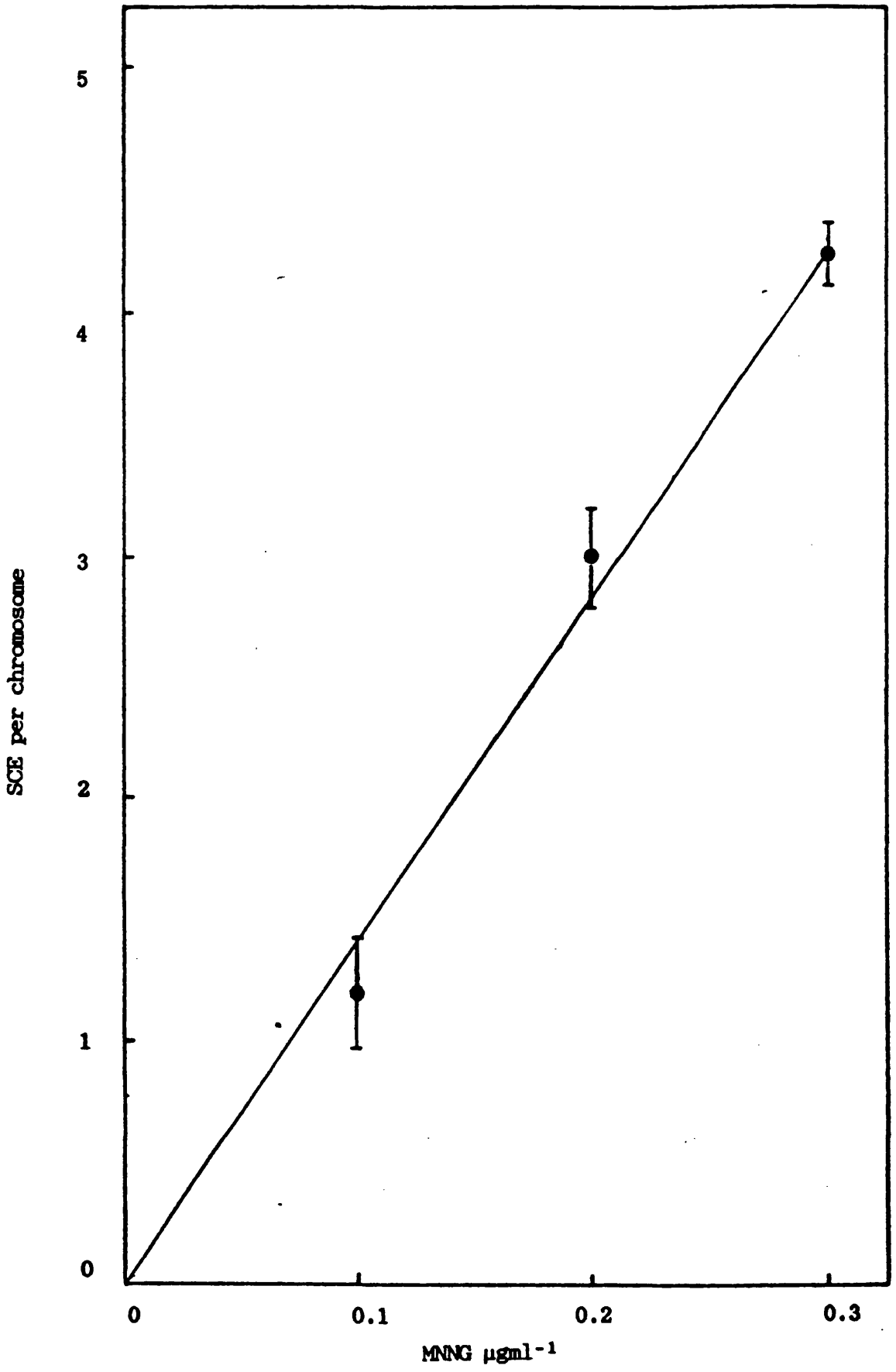


Figure 7.1. The MNNG-induced SCE frequencies in CHO-K1 cells grown in Hams F10 + 5% FCS lacking thymidine. The background SCE frequencies have been subtracted at each dose level. (n=3 ± S.E.)

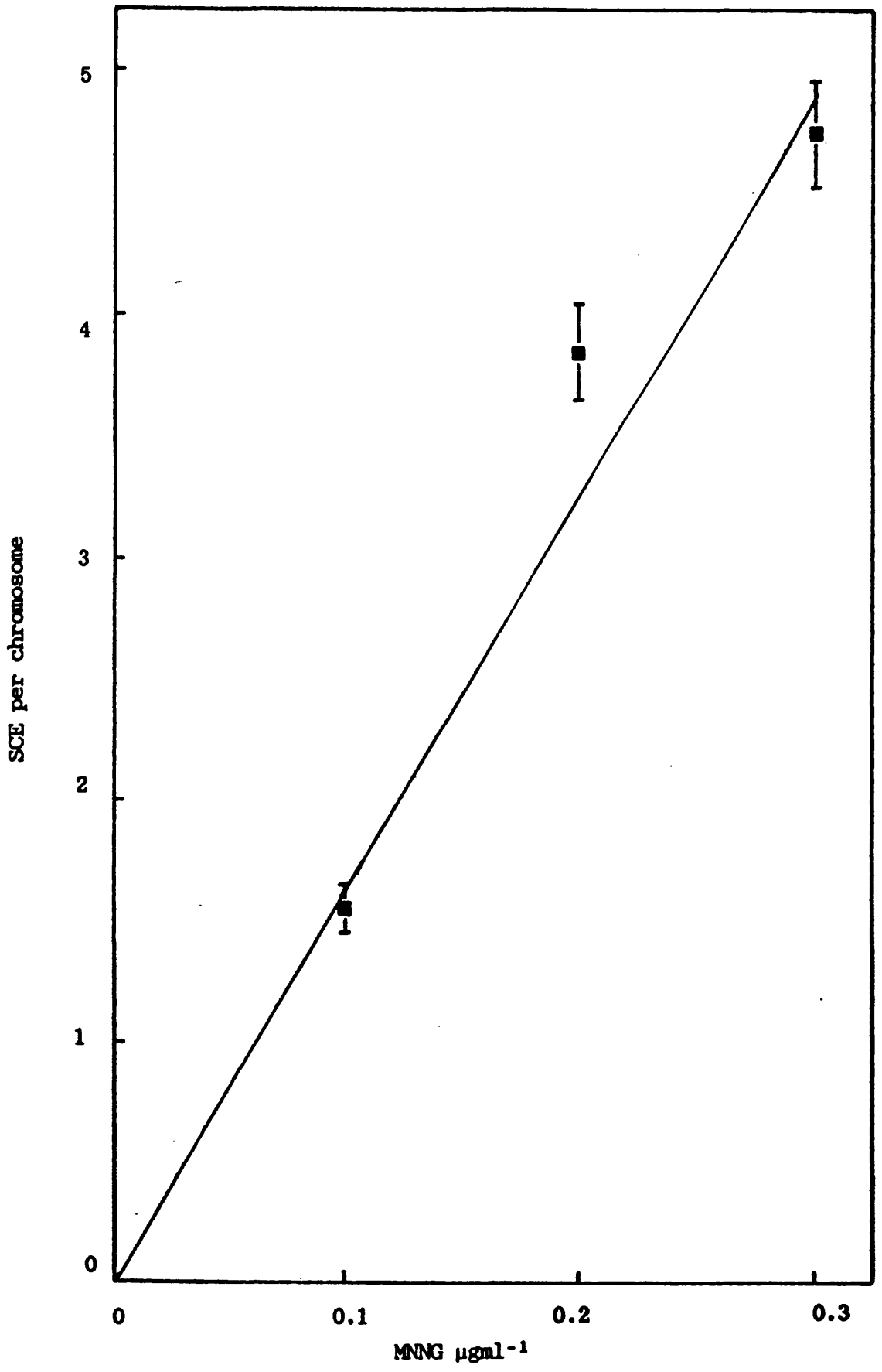


Figure 7.2. The MNNG-induced SCE frequencies in V79-379A cells grown in Hams F10 + 5% FCS lacking thymidine. The background SCE frequencies have been subtracted at each dose level. (n=3 ± S.E.)

Table 7.1. The MNNG-induced SCE frequencies in CHO-K1 cells grown in Hams F10 + 5% FCS lacking thymidine.

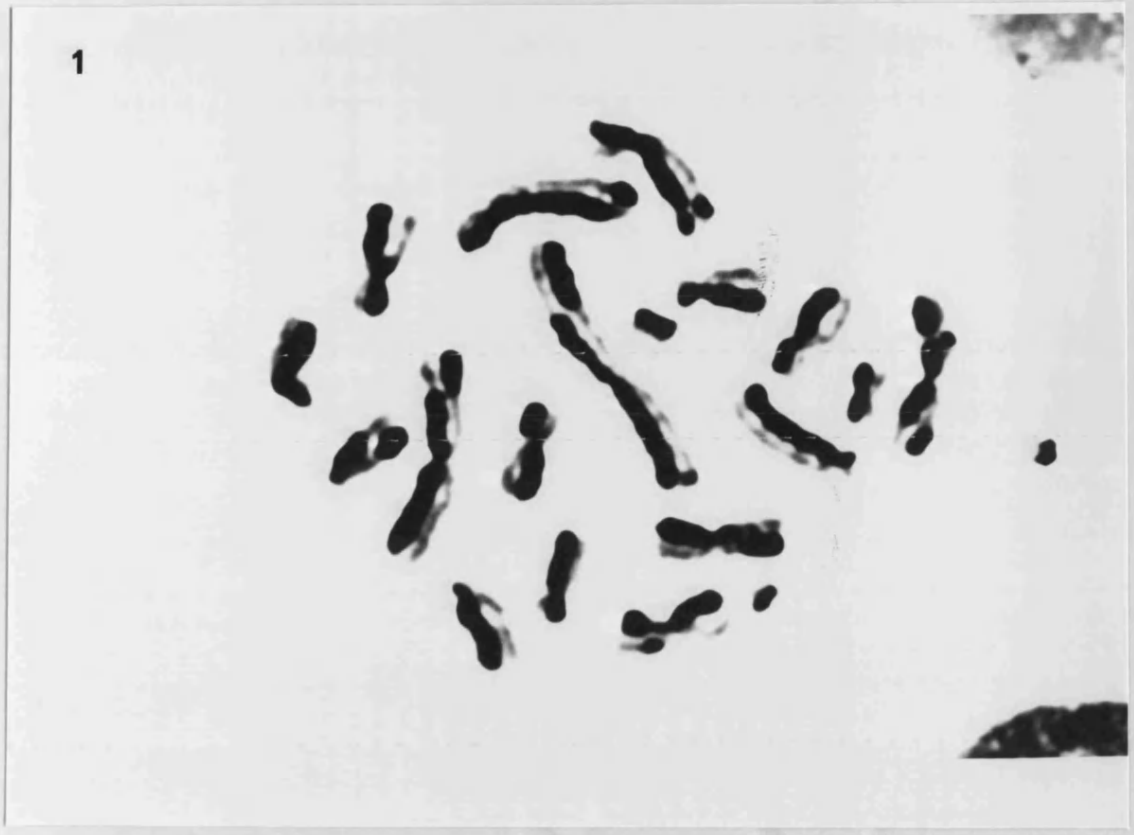
MNNG μgml^{-1}	Total cells scored.	Number of chromosomes*.	Number of SCE.	SCE per cell.	Mean SCE per cell±S.E.	SCE per chromosome.	Mean SCE per chrom.±S.E	Induced SCE per chromosome	Mean induced SCE per chrom.±S.E.
0	7	136	59	8.43		0.43			
	7	137	60	8.57	9±1	0.44	0.44±0.1	0	0
	5	98	43	8.60		0.44			
0.1	7	142	220	31.43		1.55		1.11	
	7	139	238	34.00	32±3	1.71	1.66±0.1	1.27	1.16±0.2
	7	140	220	31.43		1.56		1.12	
0.2	7	142	481	68.71		3.39		2.95	
	7	142	473	67.57	70±6	3.33	3.45±0.4	2.89	3.01±0.2
	5	101	366	73.20		3.62		3.18	
0.3	7	135	638	91.14		4.73		4.29	
	7	142	631	90.14	92±4	4.54	4.68±0.4	4.10	4.24±0.1
	7	137	635	93.29		4.77		4.33	

*Modal chromosome number = 20±1

Table 7.2. The MNNG-induced SCE frequencies in V79-379A cells grown in Hams F10 + 5% FCS lacking thymidine.

MNNG μgml^{-1}	Total cells scored.	Number of chromosomes*.	Number of SCE.	SCE per cell	Mean SCE per cell±S.E.	SCE per chromosome	SCE per chrom.±S.E.	Induced SCE per chromosome	Mean induced per chrom. ±S.E.
0	7	139	83	11.86		0.60			
0	7	141	70	10.00	11±2	0.50	0.54±0.11	0	0
0	7	139	72	10.29		0.52			
0.1	7	141	305	43.57		2.16		1.62	
0.1	7	137	297	42.43	42±4	2.17	2.09±0.20	1.63	1.55±0.1
0.1	7	140	272	38.85		1.94		1.40	
0.2	7	140	620	88.57		4.43		3.89	
0.2	7	141	618	88.29	88±1	4.38	4.38±0.13	3.84	3.85±0.2
0.2	7	139	607	86.71		4.37		3.83	
0.3	7	140	758	108.29		5.41		4.87	
0.3	7	139	716	102.29	106±9	5.15	5.31±0.12	4.61	4.76±0.2
0.3	7	139	743	106.14		5.35		4.81	

*Modal chromosome number = 20±1.



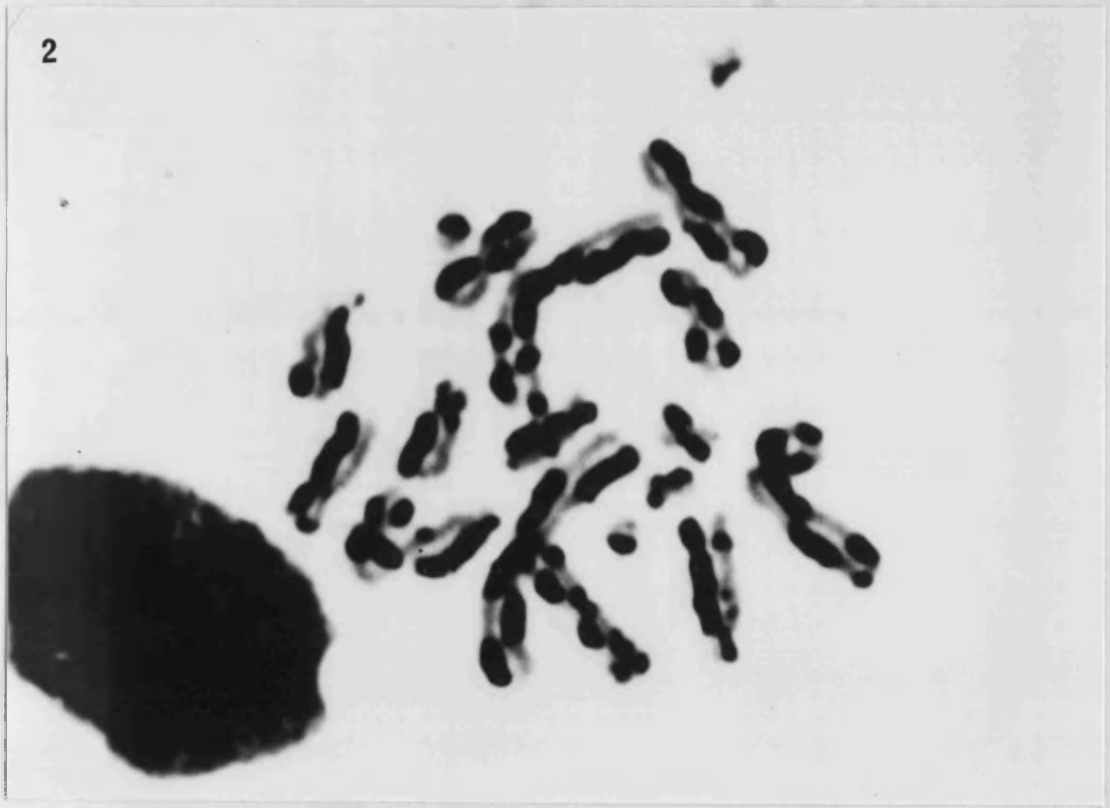


Plate 7.2. Metaphase spreads of CHO-K1 cells grown in Hams F10 + 5% FCS lacking thymidine in the presence of 10 μM BrdU, stained by the FPG technique. (1) = control, treated with vehicle, showing 12 SCE, and (2) treated with 0.1 $\mu g ml^{-1}$ MNNG showing 34 SCE.

7.4.2. MNNG-induced SCE in CHO-K1R and V79-379AR cells.

Dose-response curves for both CHO-K1R and V79-379A/R cells were constructed following the protocol outlined in 7.4.1. for CHO-K1 and V79-379A cells. The results are presented graphically on Fig. 7.3.a and b, for CHO-K1R and V79-379AR cells respectively. Tables 7.1. and 7.2. illustrate the data obtained for a series of SCE experiments by presenting data for the MNNG-induced SCE frequencies for CHO-K1 and V79-379A cells. The most obvious difference between resistant and parental cells is the presence of a double chromosome set i.e. they are polyploid. This is illustrated in Plates 7.3. and 7.4. CHO-K1 and V79-379A cells have modal chromosome numbers of 20±2 chromosomes per cell, the MNNG-resistant clones have 39±2 chromosomes per cell.

Although the resistant cells have exactly double the number of chromosomes of their parental cells, this is not matched in terms of SCE induction. The induced SCE per chromosome of CHO-K1 and CHO-K1R are similar at a dose level of 0.1 μgml^{-1} MNNG. Doses of 0.2 and 0.3 μgml^{-1} MNNG are very different, with an approximately two-fold lower level of induced SCE in CHO-K1R cells than CHO-K1 cells at the 0.3 μgml^{-1} level. This is also true of V79-379A and V79-379AR. It has been suggested that the presence of an active O^6MGMT gene will elicit lower induced-SCE than cells deficient of this gene (Bignami *et al.*, 1987; Ishizaki *et al.*, 1987; White *et al.*, 1986). However, the results from 6.5.2, where the demethylation ability of both CHO-K1R and V79-379AR were assessed, suggest the absence or inactivity of this gene.

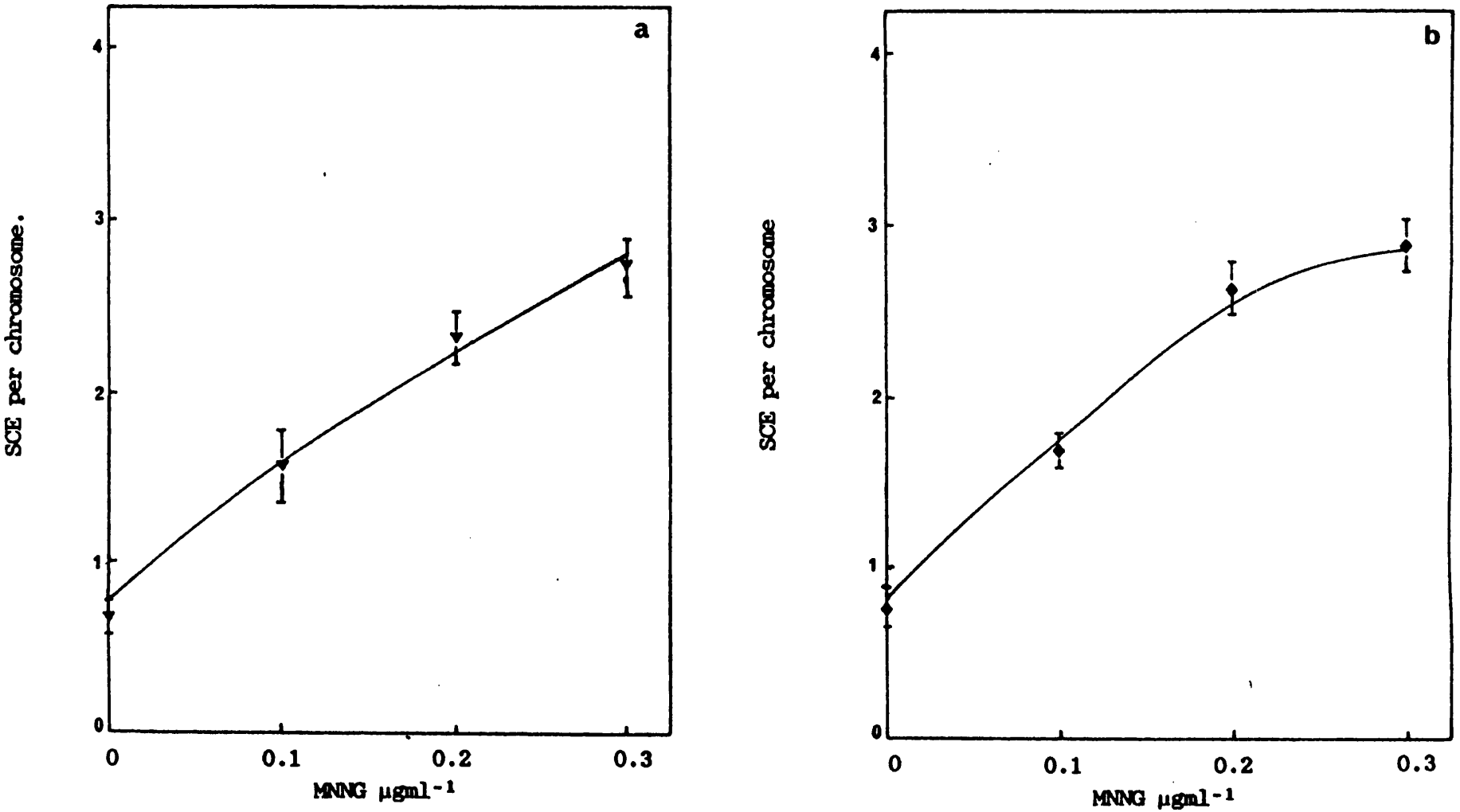


Figure 7.3. The MNNG induced SCE frequencies in CHO-K1R cells, (a), and V79-379AR cells, (b), grown in Hams F10 + 5% FCS lacking thymidine. Open and closed symbols represent pre-treated (n=2 ± S.E.) and control cells (n=3 ± S.E.) respectively.

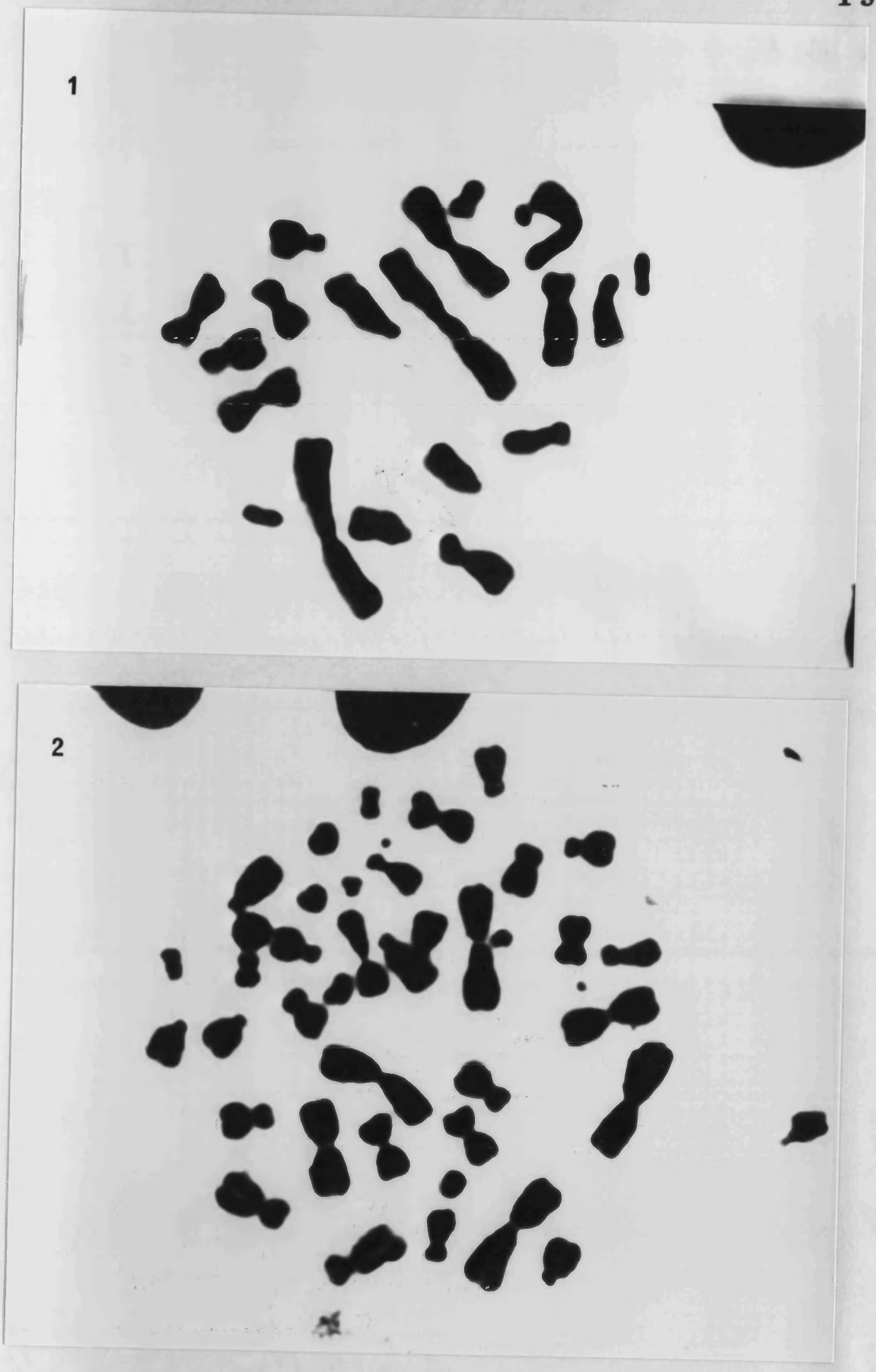


Plate 7.3. Metaphase spreads of a CHO-K1 cell, (1), and a CHO-K1R cell, (2), grown in Hams F10 + 5% FCS and stained with 5% Giemsa. Chromosome numbers of 20 and 40 are illustrated respectively

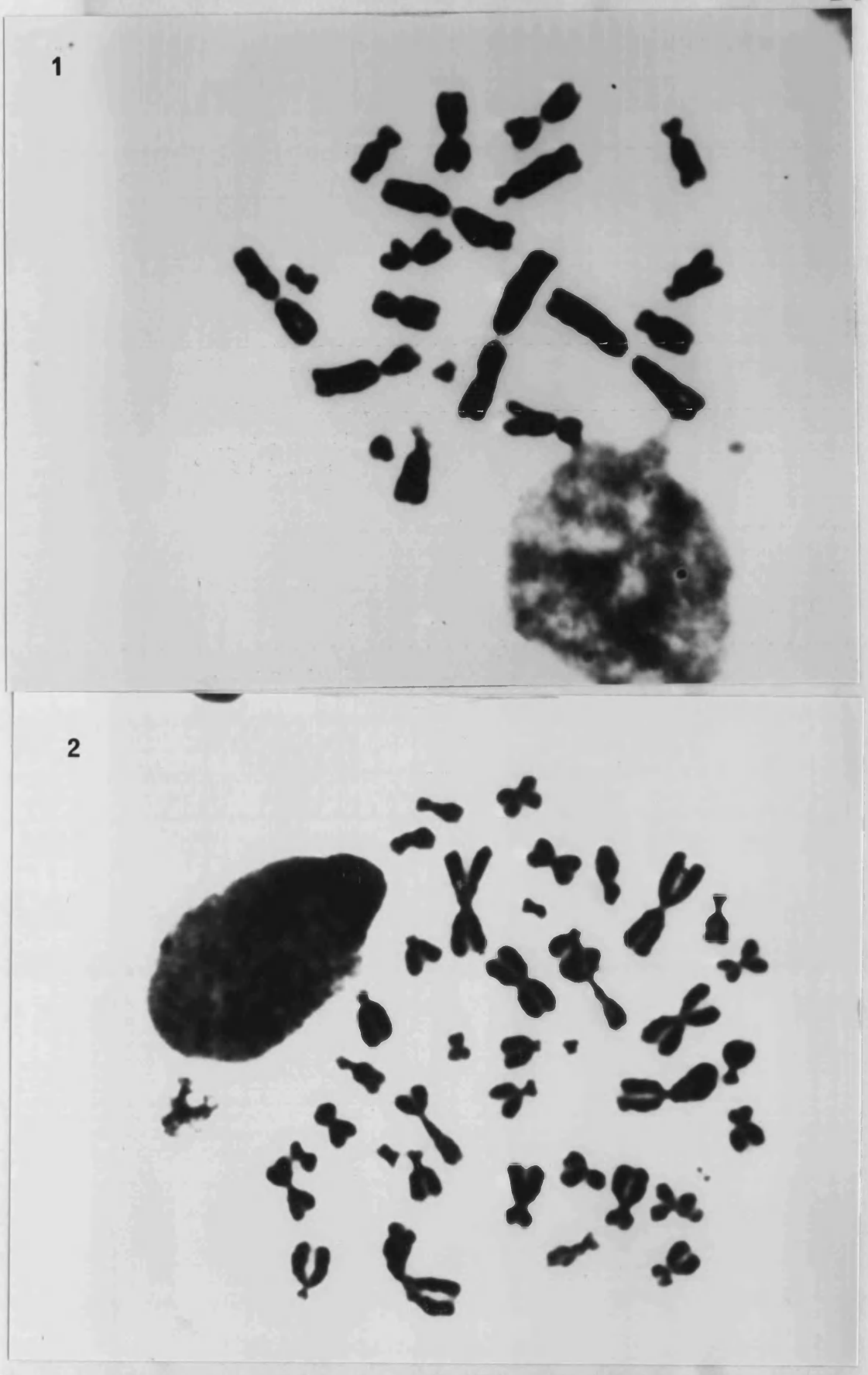


Plate 7.4. Metaphase spreads of a V79-379A cell, (1), and a V79-379AR cell, (2), grown in Hams F10 + 5% FCS and stained with 5% Giemsa. Chromosome numbers of 19 and 37 are illustrated, respectively.

7.4.3. The effect of adaptive pre-treatment on the MNNG-induced SCE frequencies in CHO-K1, CHO-K1R, V79-379A and V79-379AR cells.

Two days prior to each experiment cultures of each cell line were initiated by inoculating $5x10^5$ cells into 150ml culture bottles containing 15ml Hams F10 + 5% FCS, gassed with 5% CO_2 in air and incubated at 37.5°C. After 48 hours growth cells were dispersed with trypsin (2.6.2.) and the cell density determined (2.6.3.). For each cell line duplicate $25cm^2$ flasks were inoculated with $4x10^5$ cells containing 5ml Hams F10 + 5% FCS plus 0.01 μgml^{-1} MNNG (t=-48). The flasks were gassed with 5% CO_2 in air and incubated at 37.5°C for 6 hours. After this time (t=-42) the media was discarded and replaced with 5ml of fresh, pre-warmed Hams F10 + 5% FCS containing 0.01 μgml^{-1} MNNG. This procedure was repeated every 6 hours until t=0 was reached (Fig.7.7.). At t=0 cells were harvested and $4x10^5$ cells inoculated into a number of $25cm^2$ T/C flasks each containing 5ml of Hams F10 + 5% FCS lacking thymidine (2.3.5.(ii)) i.e duplicate flasks for control and each mutagen dose level. After a two hour incubation period 10μM BrdU was added. Test or control treatments were also introduced to the flasks at this stage. Following a further 30 hour incubation 5μM colchicine was added and 2 hours later the cells were harvested by the procedure outlined in 7.3.1. Spreads of each cell line at each mutagen dose level were prepared and stained as previously detailed in 7.3.1. and 7.3.2. and SCE were scored (7.3.3.). The results obtained are presented graphically in Figs.7.4., 7.5. and 7.6. Tables 7.3. and 7.4. show the results obtained for CHO-K1 and V79-379A numerically.

Both CHO-K1 and V79-379A cell lines exhibit reduced SCE

frequencies following adaptive pre-treatment but only at MNNG challenge doses of 0.2 and 0.3 $\mu g ml^{-1}$. Background levels of SCE are considerably higher in pre-treated cells than untreated controls with values of 2.21 vs 0.44 SCE per chromosome for CHO-K1 and 1.97 vs 0.54 SCE per chromosome for V79-379A cells. Figures 7.4. and 7.5. show the effect of pre-treatment on both cell lines, the results expressed as induced SCE per chromosome following subtraction of the relevant background (vehicle control) count (Thompson *et al.*, 1982).

CHO-K1R and V79-379AR cells show little difference between the MNNG-induced SCE frequencies of pre-treated and untreated cells (Fig.7.6). The background levels of SCE also remain unchanged following pre-treatment.

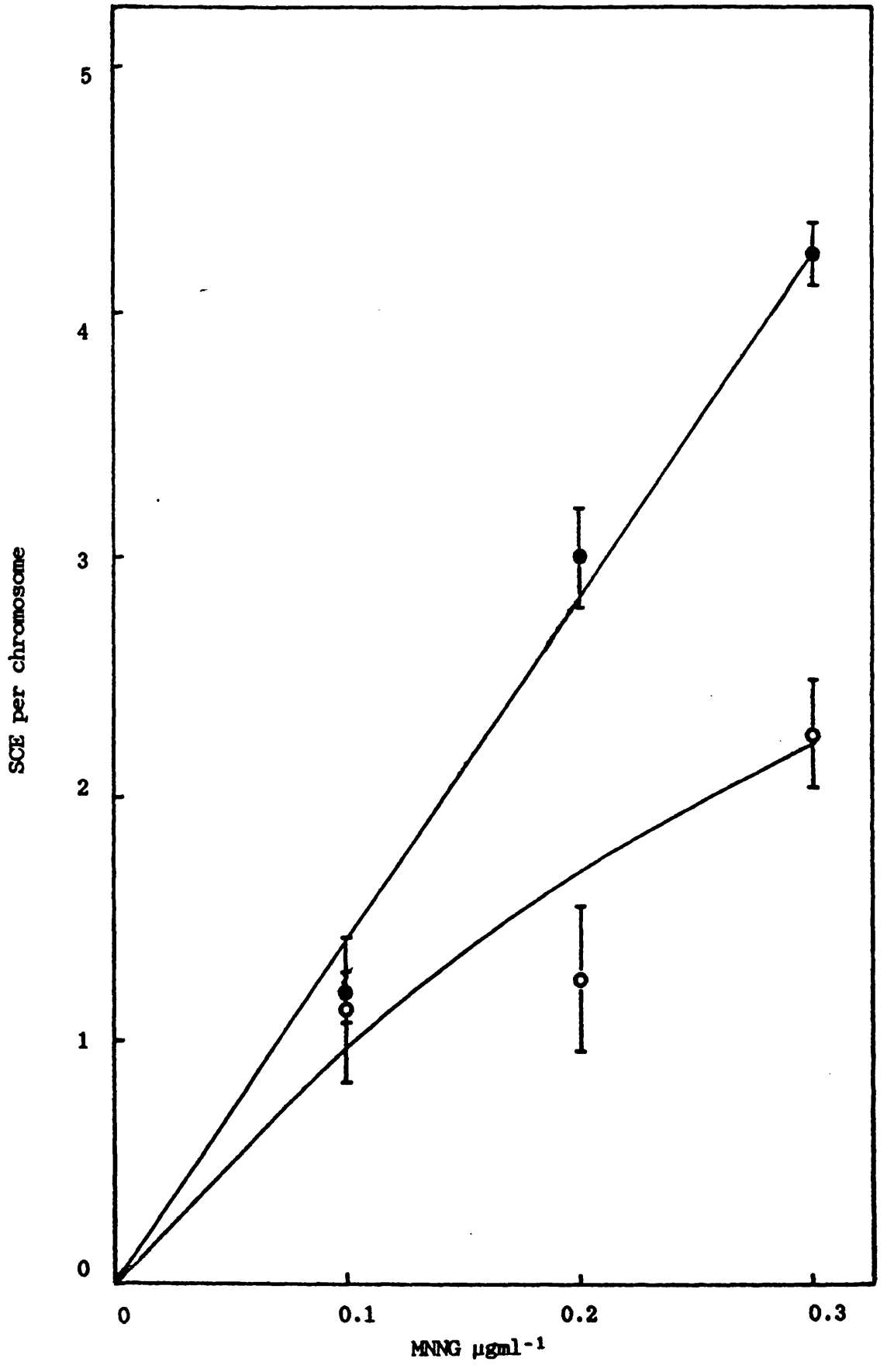


Figure 7.4. The effect of adaptive pre-treatment, by Protocol 2, on the MNNG-induced SCE frequencies in CHO-K1 cells grown in Hams F10 + 5% FCS lacking thymidine. Open and closed symbols represent pre-treated cells (n=2 ± S.E.) and control cells (n=3 ± S.E.) respectively.

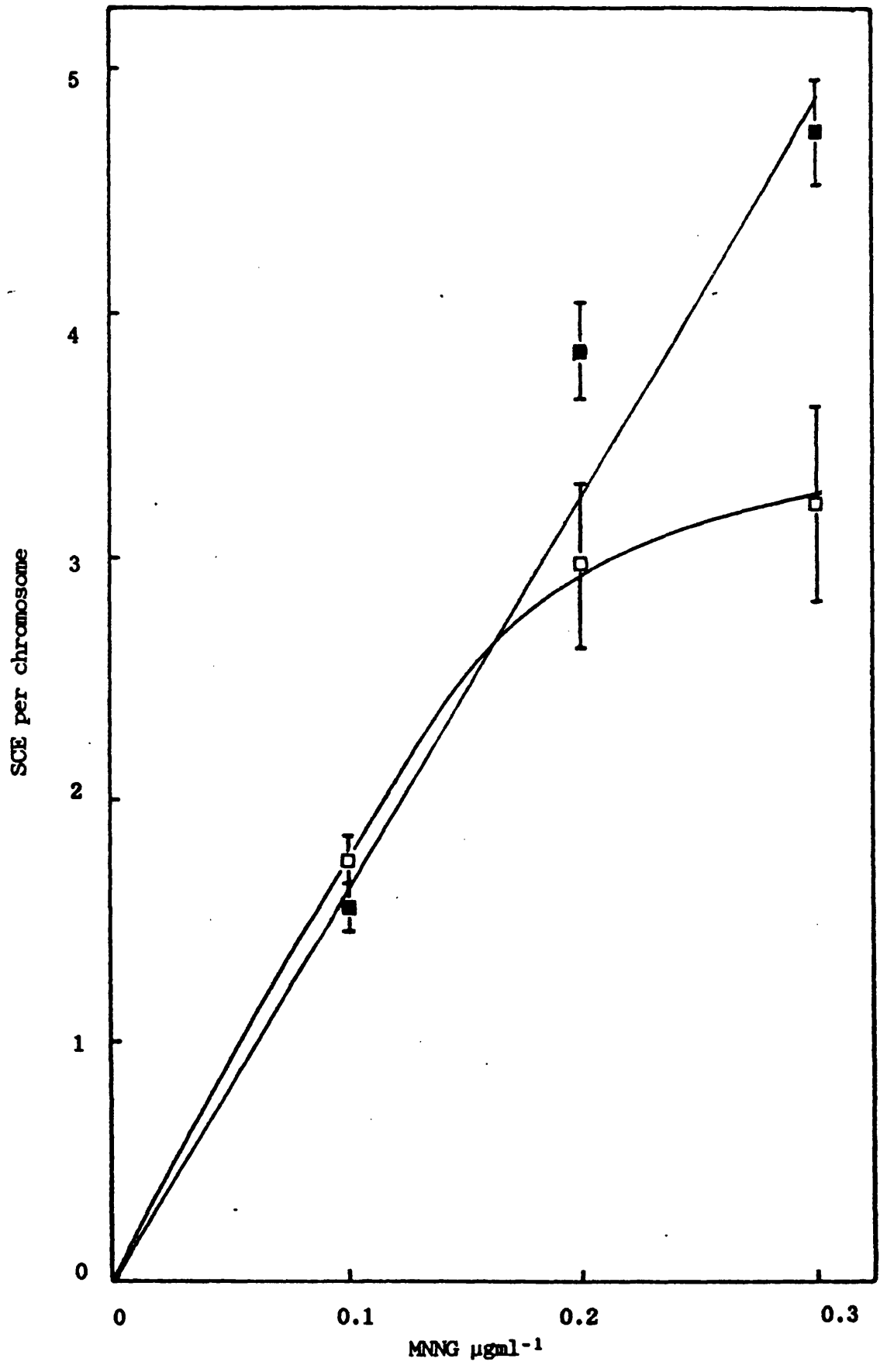


Figure 7.5. The effect of adaptive pre-treatment, by Protocol 2, on the MNNG-induced SCE frequencies in V79-379A cells grown in Hams F10 + 5% FCS lacking thymidine. Open and closed symbols represent pre-treated cells (n=2 ± S.E.) and control cells (n=3 ± S.E.) respectively.

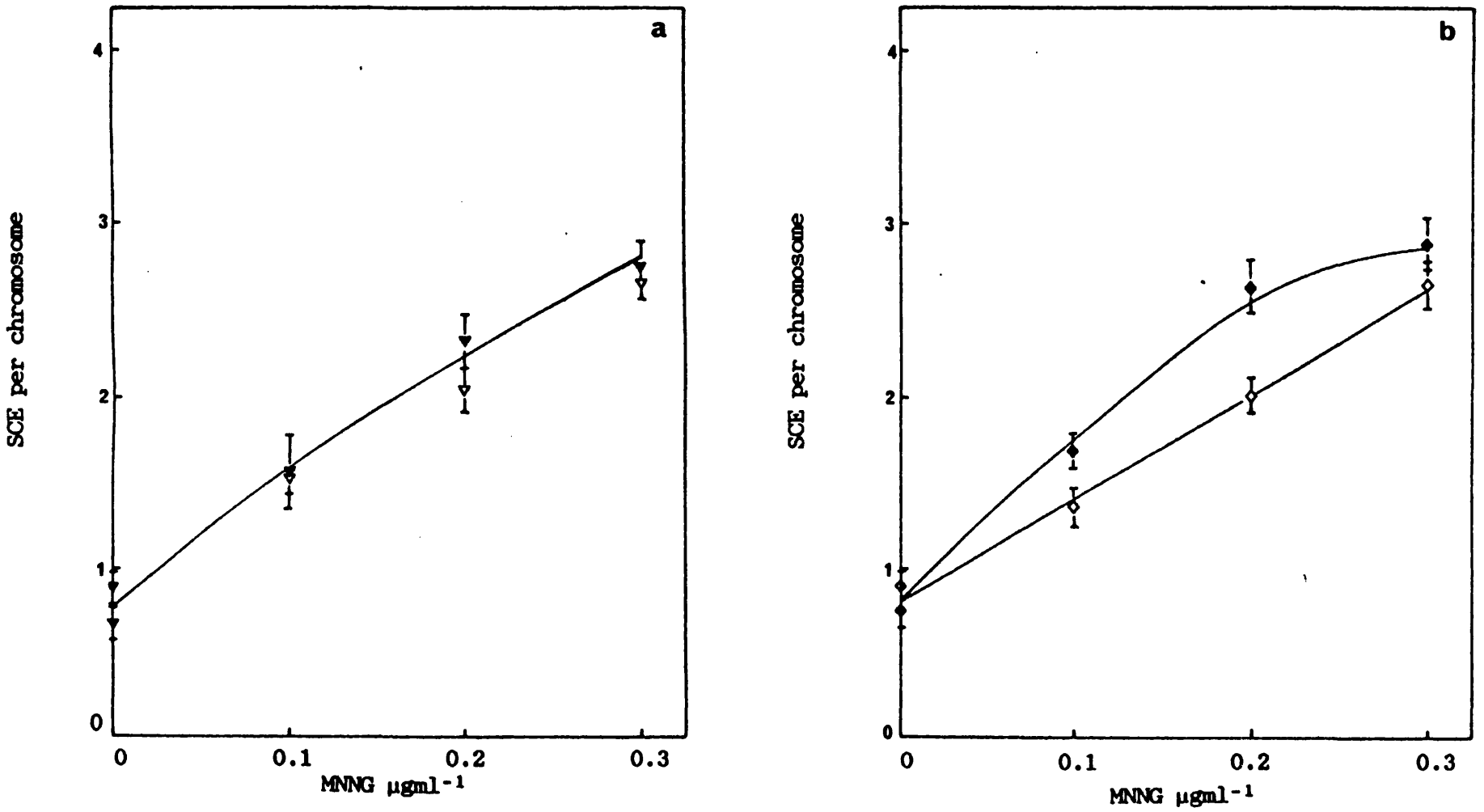


Figure 7.6. The effect of adaptive pre-treatment, by Protocol 2, on the MNNG-induced SCE frequencies in CHO-K1R cells, (a) and V79-379AR cells, (b), grown in Hams F10 + 5% FCS lacking thymidine. Open and closed symbols represent pre-treated (n=2 ± S.E.) and control cells (n=3 ± S.E.) respectively.

Table 7.3. The effect of adaptive pre-treatment, by Protocol 2, on the MNNG-induced SCE frequencies in CHO-K1 cells grown in Hams F10 + 5% FCS laking thymidine.

MNNG μgml^{-1}	Total cells scored.	Expt. N°.	Number of chromosomes.	Number of SCE.	SCE per cell.	SCE per chromosome	Mean SCE per chrom. ±S.E.	Induced SCE per chrom.	Mean induced SCE per chrom. ±S.E.
0	7	1	414	312	44.6	2.21			
	7	2	137	301	43.0	2.19	2.23±0.31	0	0
	7	2	282*	647	92.4	2.29			
0.1	6	1	121	369	61.5	3.05		0.82	
	6	2	116	359	59.8	3.09	3.29±0.11	0.86	1.14±0.3
	5	2	160*	635	127.0	3.96		1.73	
0.2	6	1	122	460	76.7	3.77		1.54	
	3	2	60	225	75.0	3.75	3.49±0.27	1.52	1.26±0.27
	5	2	196*	577	115.4	2.94		0.71	
0.3	6	1	122	538	89.7	4.41		2.18	
	6	2	118	577	96.2	4.89	4.49±0.21	2.66	2.27±0.21
	6	2	242*	1011	168.5	4.18		1.95	

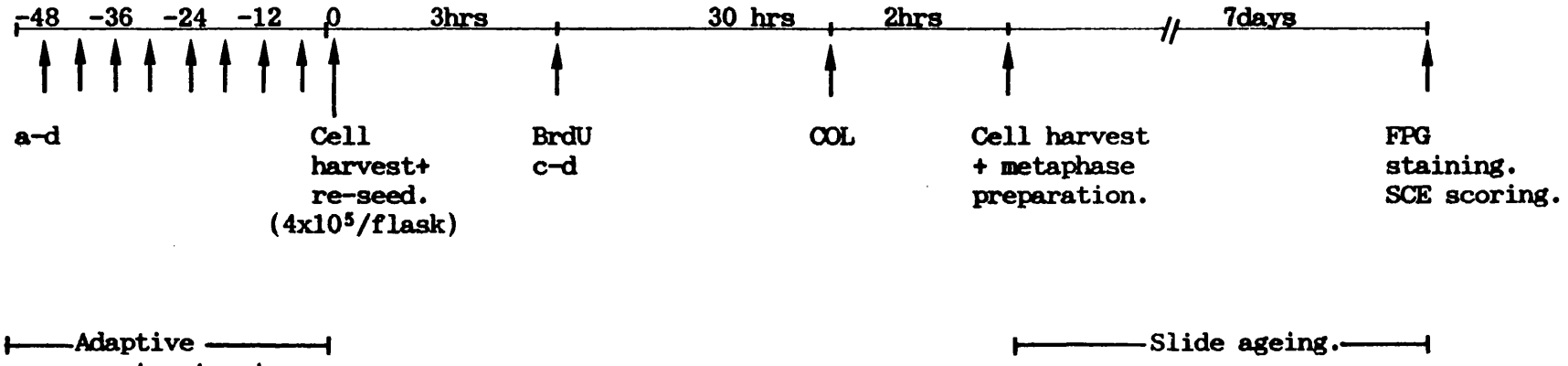
*Polyploid cells (49% of cells scored in Expt.2.)

Table 7.4. The effect of adaptive pre-treatment, by protocol 2, on the MNNG-induced frequencies in V79-379A cells grown in Hams F10 + 5% FCS laking thymidine.

MNNG $\mu g ml^{-1}$	Total cells scored.	Expt. N°.	Number of chromosomes	Number of SCE.	SCE per cell.	SCE per chromosome	Mean SCE per chrom. ±S.E.	Induced SCE per chrom.	Mean induced SCE per chrom. ±S.E.
0	6	1	124	228	38.0	1.83			
	6	2	119	240	40.0	2.02	1.97±0.10	0	0
	7	2	279*	574	82.0	2.06			
0.1	6	1	121	431	71.8	3.56		1.59	
	6	2	118	449	74.8	3.81	3.65±0.10	1.84	1.75±0.10
	6	2	239*	861	143.5	3.60		1.82	
0.2	6	1	120	621	103.5	5.18		3.21	
	6	2	116	616	102.7	5.31	4.94±0.31	3.34	2.97±0.31
	6	2	239*	1033	172.2	4.32		2.35	
0.3	6	1	120	698	116.3	5.82		3.85	
	6	2	117	615	102.5	5.26	5.19±0.39	3.29	3.22±0.39
	4	2	161*	722	180.5	4.48		2.51	

*Polyploid cells (34% of cells scored in Expt.2.)

Figure 7.5. Diagrammatical summary of the protocol used to assess the effect of adaptive pre-treatment on the MNNG-induced SCE in CHO-K1, CHO-K1R, V79-379A and V79-379AR cells.



a-d = adaptive dose - 0.01 $\mu g ml^{-1}$ MNNG.
c-d = challenge dose - 0, 0.1, 0.2 and 0.3 $\mu g ml^{-1}$ MNNG.
BrdU = 10μM 5-bromodeoxyuridine.
COL = 5μM colchicine.
FPG = Fluorescence plus Giemsa (7.3.2.).

7.5. Conclusions.

From the results presented in this chapter the following conclusions may be drawn.

1) Sister chromatid exchanges (SCE) can be induced by MNNG in CHO-K1, CHO-K1R, V79-379A and V79-379AR cells and visualised by differential staining of the chromatid arms (7.3.1. and 7.3.2.)
2) Both CHO-K1 and V79-379A were found to have a modal chromosome number of 20±2, whereas CHO-K1R and V79-379AR cells were polyploid with a modal number fo 39±2 (Plates 7.3. and 7.4.).
3) MNNG, in the concentration range 0.1 to 0.3 μgml^{-1} induced SCE in a dose dependent fashion for CHO-K1, CHO-K1R, V79-379A and V79-379AR cells (Figs.7.1., 7.2. and 7.3.), to a maximum frequency of 4.76 SCE per chromosome.
4) The background (vehicle control) SCE frequencies were 0.44 SCE per chromosome for CHO-K1, 0.67 SCE per chromosome for CHO-K1R, 0.54 SCE per chromosome for V79-379A and 0.75 SCE per chromosome for V79-379AR cells.
5) Adaptive pre-treatment of CHO-K1 and V79-379A cells lowered the induced SCE frequencies at MNNG challenge levels of 0.2 and 0.3 μgml^{-1} but not at 0.1 μgml^{-1} Figs.7.4. and 7.5.).
6) Adaptive pre-treatment increased the background SCE levels over control for CHO-K1 (0.45 to 2.23 SCE per chromosome) and V79-379A cells (0.54 to 1.97 SCE per chromosome) (Tables 7.3. and 7.4.).
7) CHO-K1 and V79-379A cell populations exhibited a high percentage of polyploid cells i.e. 49% and 34% respectively, following adaptive pre-treatment (Tables 7.3. and 7.4.).
8) Neither the background nor the induced SCE frequencies of CHO-K1R and V79-379AR cells were altered by adaptive

pre-treatment.

These conclusions will be discussed in Chapter 8.

CHAPTER 8.1 DISCUSSION.

The adaptive response to alkylating agents is an inducible form of DNA repair acting on alkylation damage. When *E.coli* cultures are exposed to low concentrations of alkylating agents, such as N-methyl-N'-nitrosoguanidine (MNNG), they become resistant to the cytotoxic and mutagenic effects of high concentrations of these compounds. Two enzymes are associated with this response and are induced by adaptive pre-treatment i.e. 3-methyladenine-DNA -glycosylase II (3-MAG), which removes 3-methyladenine (3MA), 7-methylguanine (7MG) and 3-methylguanine (3MG) from alkylated DNA, and O^6-methylguanine-DNA-methyltransferase (O^6MGMT), which de-methylates O^6-methylguanine (O^6MGMT). In *E.coli*, under adaptive conditions, the cellular levels of these enzymes increase 20 and 100-200 fold, respectively. The repair of O^6MG prevents this lesion causing G:C to A:T transition mutations whereas the removal of 3MA and 3MG prevents cell killing. The presence of these enzymes in mammalian cell systems has been reported by a number of authors (Cathcart and Goldthwait, 1981; see also Table 1.2.). Possession of such enzymes suggests that mammalian cells have the capability to express an adaptive response if induced by adaptive pre-treatment. Evidence for the existence of a response, analogous to that of *E.coli*, has so far been conflicting (see Chapter 1). Much work has been carried out using *in vitro* cell culture systems from which two relevant questions have arisen; 1) are the discrepancies that have been found due to the different experimental protocols used and 2) are the contrasting results that have been reported, due to the different cell types used ?. The overall aim of this project, therefore, was to attempt to answer these questions by choosing two cell lines, common to the

previous studies, and then subjecting them to adaptive pre-treatments following two different protocols. Each protocol was chosen by the virtue of its proven capability to induce the adaptive response in one or both cell lines. The choice of each assay system used in this project was also based on previous studies which employed one or more of these biological end-points. The effect of MNNG pre-treatment on each cell line was assessed by changes in MNNG-induced cytotoxicity and mutation to ouabain resistance, growth parameter changes, sister chromatid exchanges and by indirect enzyme analysis of cell-free extracts.

Single plated CHO-K1 cells, exposed to increasing levels of MNNG, exhibited a dose-dependent decrease in survival as shown in Fig.3.1. The assay employed was demonstrated to be reproducible for both CHO-K1 and V79-379A cell lines (Figs.3.2. and 3.3.). For each cell line, plating efficiencies for control cells were within the range 70 to 95% and were similar to the plating efficiencies found previously by other workers using Chinese hamster cell lines. (Bradley *et al.*, 1981; Dewdney, 1982). Cell killing in both cell lines was seen to be bi-phasic when cell survival was plotted, on a log scale, as a function of MNNG dose. The curves show an immediate exponential reduction in cell survival with mutagen doses up to 0.05 $\mu g ml^{-1}$ MNNG. At mutagen concentrations between 0.05 $\mu g ml^{-1}$ and 0.3 $\mu g ml^{-1}$ MNNG there is a further exponential reduction in cell survival but the rate of reduction is slower. If both exponential portions of the curve are extrapolated the inflection point of the curve can be derived from the intersection of the two lines. For CHO-K1 cells the curve inflection is observed at an MNNG concentration of 0.05 $\mu g ml^{-1}$ and

a survival level of 4% and for V79-379A cells the values are 0.1 μgml^{-1} and 2% survival. A bi-phasic curve could indicate the presence of two sub-populations of cells, each differing in their sensitivity to MNNG. The intercept with the y-axis of the latter extrapolation line, for the second exponential phase of the curve, gives an approximation of the percentage of the more resistant sub-population. From Figs.3.2. and 3.3. the population of CHO-K1 cells was shown to be comprised of 6% resistant cells and the population of V79-379A cells contained 4% resistant cells. Bi-phasic dose-survivor curves have been reported by a number of authors using a variety of Chinese hamster cell lines. Barranco and Humphrey, (1971), reported the existence of an MNNG-resistant sub-population of CHO cells that comprised 3% of the overall population. Wilson, (1985) reported that 5% of a population of V79-379A cells were resistant to MNNG. Bi-phasic survivor curves, similar to those shown in Figs.3.1. and 3.2. were reported by Goth-Goldstein and Hughes, (1987b), following treatment of CHO-9 cells with either MNNG or MNU. For each mutagen, extrapolation of the second exponential portion of the survivor curve showed that 4% of the overall population were more resistant to MNNG whilst 10% were resistant to MNU. When this cell line was treated with MMS and ENU, however, there was no indication of resistant sub-populations, since cell killing was a simple exponential decrease in survival with this mutagen, following a slight shoulder region. This may suggest the production of bi-phasic curves is dependent on the type of mutagen used. However, this does not seem to be the case, since the MNNG-induced cell killing of C3H $10T^1/_2$ cells, another rodent fibroblast line, was shown to be a simple dose-dependent reduction in survival with MNNG dose,

rather than a bi-phasic response (Grisham and Smith, 1984).

Carver *et al.*, (1979), estimated the cytotoxic potency of a number of mutagens by comparing the relative D_{37} values. The D_{37} value is defined as the dose required to kill 63% of the cell population (37% survival) and is considered to be a valid measure of cytotoxic potency. From the dose-response data plotted for MNNG treated CHO-K1 cells, a D_{37} value of 0.021 μgml^{-1} was derived (Fig.3.2.). This is similar to values previously obtained for this cell line by Dewdney, (1982) (D_{37} = 0.035 μgml^{-1}) and Wilson, (1985) (D_{37} = 0.021 μgml^{-1}). When compared to parental CHO cells, however, (Barranco and Humphrey, 1971, D_{37} = 0.07 μgml^{-1}; Kao and Puck, 1968, D_{37} = 0.44 μgml^{-1}) single plated CHO-K1 cells are found to be more sensitive to the toxic effects of MNNG. A similar relative sensitivity is seen when considering V79-379A cells. The dose-response data plotted for these cells gave a D_{37} value of 0.012 μgml^{-1} (Fig.3.3.) which agrees with the findings of Wilson, (1985), who also used V79-379A cells (D_{37} = 0.011 μgml^{-1}), but not with other workers with V79 cells. Chu and Malling, (1968) reported a D_{37} value of 0.74 μgml^{-1} and Peterson *et al.*, (1979) a D_{37} value of 1.03 μgml^{-1}. The reason(s) for the sensitivity of CHO-K1 and V79-379A cells compared to their parental cells still remains to be elucidated. However, the degradation of solid MNNG during the course of the studies was discounted by Wilson, (1985) who compared the UV absorption spectra of MNNG, used throughout her studies, with a newly acquired MNNG batch, finding no difference in peak maxima.

The adaptive pre-treatment dose, used throughout these studies, was chosen on the basis of the minimal effect on plating efficiency (i.e. survival) of both CHO-K1 and V79-379A cell lines.

MNNG concentrations in the range 0 - 0.02 μgml^{-1} were used to determine the point at which a measurable decrease in cell survival occured. Fig. 3.4. illustrates the effects of these concentrations on the survival of each cell line. From these results the adaptive pre-treatment dose was chosen as 0.01 μgml^{-1} MNNG, since it was the highest concentration tolerated by each cell line before cytotoxicity was evident i.e. 0.01 μgml^{-1} is non-toxic. This mutagen concentration exactly matches that used by Samson and Schwartz, (1980) and is similar to the MNNG pre-treatment doses of 0.011 μgml^{-1} used by Kaina, (1983b) and 0.0102 μgml^{-1} used by Frosina *et al.*, (1984) on V79 cells. Figure 3.4. also indicates the presence of a small shoulder to the dose-survivor curves of both CHO-K1 and V79-379A cells which was not evident in Figs.3.2. and 3.3. The shoulder region of a survivor curve produced by low doses of a chemical has been considered evidence of a cells capacity to accumulate or repair some damage before that damage becomes lethal (Elkind and Sutton, 1959; Munson and Goodhead, 1977).

The initial approach adopted for these studies involved the use of two adaptive protocols, one involving a single non-toxic pre-treatment dose (Protocol 1) and the other, multiple mutagen doses (Protocol 2) (Fig.3.5.). Kaina, (1982) subjected V79-C10 cells to a single, non-toxic dose of either MNU or MNNG and challenged these cells six hours later with high doses of the same agent. These cells were shown to have increased survival, reduced mutation to $6\text{-}TG^R$ and reduced chromosomal aberrations following this pre-treatment, when compared to untreated control cells. Samson and Schwartz (1980), using a multiple MNNG dose pre-treatment protocol (Protocol 2), reported an enhanced cell

survival of CHO cells to subsequent MNNG challenge. These authors also reported a decrease in the MNNG-induced SCE frequency, in pre-treated cells, compared to control cells.

Application of each pre-treatment protocol to CHO-K1 and V79-379A cells was carried out and the effect of pre-treatment assessed, initially, in respect to changes in cell survival only. CHO-K1 cells seeded, pre-treated and challenged with MNNG after 6 hours incubation showed no difference in survival when the D_{37} values of control and pre-treated cells are compared (Fig.3.6.a) i.e. 0.06 $\mu g ml^{-1}$ MNNG.

Treatment of V79-379A cell by Protocol 1 produced an enhanced survival of pre-treated cells (D_{37}=0.021 $\mu g ml^{-1}$ MNNG) over untreated control cells (D_{37}=0.011 $\mu g ml^{-1}$ MNNG) (Fig.3.6.b). This result agrees with the findings of Kaina, (1982) who also found a 2-fold increase in cell survival using this pre-treatment protocol. The D_{37} value of the control cells is similar to that previously shown for V79-379A cells in 3.2.2. i.e. 0.012 $\mu g ml^{-1}$ MNNG.

Pre-treatment by Protocol 2 of both CHO-K1 and V79-379A cells resulted in an enhanced cell survival to subsequent mutagen challenge (Figs.3.7. and 3.8.). Pre-treated CHO-K1 cells exhibited a 3-fold enhancement of survival over control, and V79-379A a 6.8-fold enhancement.

The observed enhancement of survival of V79-379A cells following pre-treatment by Protocol 1, and of both cell lines by Protocol 2, may be explained in a number of ways:

a) Pre-treatment has altered in some way, the cellular mechanisms that deal with alkylation damage either by preventing alkylation damage or by repairing alkylation damage more efficiently

consequently preventing cell killing. If the latter were true then one may suggest that an enzyme comparable to the 3-MAG induced during the *E.coli* adaptive response could be responsible. This theory cannot be ruled out but since activity was not assessed in these cells, during the present study, direct evidence is lacking.

b) The initial alkylations, caused by MNNG pre-treatment, could make the cells resistant to the second treatment by preventing the challenge dose from entering the cell or reaching the DNA target site. The results of Vistica, (1979) support this theory by showing that nitrogen mustard-sensitive mouse L5178Y lymphoblastoid cells had a reduced uptake of this mutagen, compared to the parental cells, which was due to an inactivation of a membrane transport system. In contrast, Schendel and Robins (1978) ruled out the possibility of diminished mutagen accessibility of adapted *E.coli* cells, by showing that the uptake kinetics of ^{14}C-MNNG in both pre-treated and control cells was similar.

c) Since the action of MNNG is stimulated by cellular thiol levels (Lawley and Thatcher, 1970) a pre-treatment induced reduction of thiol levels could inhibit the cytotoxic action of MNNG. However Laval and Laval, (1984) found that the total thiol level within rat H$_4$ cells was not modified following pre-treatment by a protocol similar to Protocol 2. Furthermore, Samson and Schwartz, (1980) showed that MNNG pre-treated CHO cells were resistant to challenge with the alkylating agents MMS and EMS, whose cytotoxicity is unaffected by thiol content.

d) Adaptive pre-treatment may have changed the cell cycle distribution and since the sensitivity of CHO cells, to MNNG, fluctuates throughout the cell cycle (Barranco and Humphrey,

1971), this change may account for the increased resistance of adaptively pre-treated cells to subsequent mutagen challenge. CHO-K1 cells were synchronised by the method of Zwanenburg, (1983) using a mitotic shake-off technique. This technique makes use of the fact that CHO-K1 cells, growing as a monolayer, round up and become less firmly attached to the growing surface when they enter mitosis. The mitotic cells were gently shaken off the monolayer and collected by centrifugation. One advantage of this method, over other techniques, is that there is no need for the addition of any chemicals to enrich the population with cells in a specific stage of the cell cycle. Zwanenburg, (1983) calculated that a suspension containing nearly 100% mitotic cells could be obtained in this way. Using this technique, to obtain a population of synchronous CHO-K1 cells, it was shown that synchrony was not induced by adaptive Protocol 2, since the MNNG dose-survivor curve for synchronised CHO-K1 cells did not match that of adaptively pre-treated unsynchronised CHO-K1 cells, but was similar to the MNNG dose-response curve of unsynchronised CHO-K1 cells (Fig.4.12.a).

e) The most likely explanation, however, in the absence of any enzymological evidence, is that pre-treatment has selected for pre-existing mutagen resistant cells rather than inducing resistance in the whole population. The existence of a more resistant sub-population is suggested from the bi-phasic dose-survivor curve produced for both CHO-K1 and V79-379A cell lines, but selection of these cells, by Protocol 1, seems unlikely since pre-treatment occurred only several hours prior to challenge. Pre-treatment by Protocol 2, however, subjected the cell populations to adaptive mutagen doses for a much longer

period, which allowed growth to occur and possible selection of pre-existing mutagen-resistant cells. If this theory is true, a pre-treatment-induced change in the proportion of each sub-population, may be an important factor in the interpretation of data obtained from adaptation experiments. Investigations into this aspect are discussed later in this chapter.

From the results above it was observed that Protocol 2 i.e. 6-hourly mutagen doses over 48 hours, caused an enhancement of challenge-induced cell killing, in both cell lines. It was decided, therefore, to use this protocol in all subsequent adaptation experiments.

The second aspect of the *E.coli* adaptive response is mutagenic adaptation. This phenomenon has been studied in both CHO and V79 systems using a variety of mutagenic end-points. Most commonly used have been the forward mutation to 6-thioguanine resistance ($6TG^R$) or ouabain resistance (Oua^R) (Durrant *et al.*, 1981; Kaina, 1982, 1983a, b; Laval and Laval, 1984; Schwartz and Samson, 1983). Reverse mutations were used during the initial studies into the bacterial adaptive response and have also been assessed during studies into the adaptive response in V79 cells i.e. $HGPRT^-$ to $HGPRT^+$ (Fox and Carlton, 1984). However, as mentioned in Chapter 4, resistance to the steroid compound ouabain was used as a genetic marker for a number of experimentally attractive reasons; 1) its capability to detect base-pair substitution mutations, which are caused by O^6MG lesions, 2) ease of assay, 3) experimental reproducibility and 4) there are few cell density effects (Arlett *et al.*, 1975). Expanding point 2, by comparison with the well used $6TG^R$ system ($HGPRT^+$ to $HGPRT^-$), the

ouabain resistance system is desirable since it has a mutant expression time of 47-49 hours whereas expression of $6TG^R$ mutants requires a 7 day expression period with the necessity to perform one or two sub-cultures during this time. Although the $6TG^R$ may be more sensitive than the Oua^R due to its ability to detect frameshift as well as base-substitution mutations, the Oua^R genetic marker was chosen for these studies since a single type of mutation was sought. The enzyme involved in bacterial mutagenic adaptation, O^6MGMT, de-methylates the potentially mutagenic lesion O^6MG to lower the number of detectable base-pair substitution mutations. In mammalian cells, induction of this enzyme during adaptive pre-treatment would lead to a decrease in these mutations, hence a system that detected only this type of mutation was used.

The mutation of CHO-K1 cells to Oua^R was assessed using an *in situ* protocol. In this type of assay cells are plated, treated with a mutagen, and left undisturbed except for the addition of the selecting agent. The alternative respreading protocol, in which cells are mutagen treated as a monolayer, then plated out, should not be used to assess mutation to Oua^R (Arlett, 1977). Trypsinisation, necessary for the dispersal of cells, has been reported to lead to the loss of newly arisen Oua^R mutants in respreading assays (Arlett, 1977). The optimum conditions for the detection of Oua^R CHO-K1 mutants, as validated by Dewdney, (1982), are similar to those of other CHO-K1 cells (Hsie *et al.*, 1981). CHO-K1 cells treated with MNNG show a dose-dependent increase in mutation frequency (Oua^R mutants per 10^6 surviving cell) up to 3000 fold over the low, spontaneous level of $1x10^{-6}$ (Table 4.1.). The reproducibility of this assay was demonstrated for CHO-K1 and

V79-379A cells (Figs.4.2. and 4.3.). Both cell lines exhibited a dose-dependent increase in mutation frequency when subjected to increasing concentrations of MNNG over the range 0.02 μgml^{-1} to 0.3 μgml^{-1} MNNG.

Both CHO-K1 and V79-379A cell lines were found to have spontaneous mutation frequencies of approximately $1x10^{-6}$ which is in good agreement with previously reported figures i.e. Ahmed, (1977), $1.8x10^{-6}$; Arlett *et al.*, (1975), $4x10^{-7}$; Lankas *et al.*, (1977), $1.7x10^{-6}$; Lever and Seegmuller, (1976), $1x10^{-6}$.

Adaptive pre-treatment by Protocol 2, of both cell lines, resulted in an apparent decrease in the MNNG-induced mutation frequencies. The results of these experiments are shown in Figs. 4.6. and 4.7. for pre-treated CHO-K1 and V79-379A cells respectively. From these Figures it can be seen that when considering differences in mutation by comparing the induced mutants at each mutagen dose there is a substantial decrease in the frequencies following adaptive pre-treatment. If, however, one compares the effect as a function of the cell killing i.e. equicytotoxic doses, pre-treated and control cells are as equally mutable as each other for both cell lines (Fig.4.8.). Since there is a linear relationship between log cell survival and log mutation frequency (Carver *et al.*, 1979; Munson and Goodhead, 1977) a 'real' decrease in mutation frequency, following adaptive pre-treatment, should result in fewer mutational events per killing event i.e a reduction in the slope value. However this is not the case. Schwartz and Samson, (1983) reported unaltered $6TG^R$ mutation frequencies after chronic pre-treatment of CHO cells. They presented the data as mutants per 10^5 clonable cells as a function of MNNG challenge dose. If they had expressed their

results as a function of survival they would have noticed that for each challenge dose the cells were more mutable than their respective control implying that accumulation of mutants during the pre-treatment period had occured. Durrant *et al.*, (1981) pre-treated V79 cells with a single dose of MNNG and compared the induced mutation frequencies of these cells with those of untreated cells and found that, on an equicytotoxic dose level, these cells were more mutable than the control cells. The authors interpreted this result as an accumulation of mutants in the surviving cells. A real reduction in the mutation frequencies, following adaptive pre-treatment, was presented by Laval and Laval, (1984) since analysis of mutation frequency vs survival showed a lower number of mutational events per killing event when rat H_4 cells were pre-treated and challenged with MNNG. When these cells were pre-treated with MMS and then challenged with either MMS or MNNG the results showed no mutagenic adaptation, but instead indicated an accumulation of mutants i.e. the cells are more mutable following pre-treatment.

The theory that pre-treatment can cause the accumulation of mutations is supported by the fact that the spontaneous mutation frequency of pre-treated cells is much higher than that of control cells i.e. 30 fold (4.2.3.). This phenomenon was also noticed by Schwartz and Samson, (1983) who reported 40 $6TG^R$ mutants per 10^5 survivors, in pre-treated cells, compared to 2 mutants per 10^5 survivors in control cells. Accumulation of mutants during the pre-treatment period is also indicated by the increase of background SCE reported in Chapter 7 for both CHO-K1 and V79-379A cells. One possible explanation for the observed reduction in mutation, frequencies following pre-treatment, is the slowing of

cellular growth, which could lead to an extended mutant expression period, ultimately producing fewer mutants. Laval and Laval, (1984) discounted this possibilty by determining the expression time of adapted and control cells, finding a maximum expression period of five days for each. Lankas *et al.*, (1979) reported that slowly growing V79 cells produced less MNNG-induced Oua^R mutants than other cells growing at the normal rate. During these experiments, cells were grown in medium containing either 2% or 10% FCS and since care was taken to allow both the fast, (10% FCS) and the slow (2% FCS) growing cells to achieve the same number of cell divisions before ouabain selection, this group concluded that it is the growth rate and not the number of cell divisions that is important in respect to the number of mutants produced.

Partial synchrony of the cells caused by adaptive pre-treatment, does not explain the apparent drop in mutation frequencies since synchronised CHO-K1 cells were shown to exhibit similar mutation frequencies to non-synchronised cells. (Fig.4.12b).

CHO-K1 and V79-379A cells exhibit 'classical' growth kinetics, growth being conventionally divided into three distinct phases i.e. lag phase, log or exponential phase and stationary phase. Growth of these cells is evaluated by calculating the population doubling time (T) which is a measure of the rate at which cells, in the exponential phase, can double their number. Measurement of this parameter is useful since it gives an indication as to the general state of growth of the cell population. Following adaptive pre-treatment CHO-K1 cells showed a population doubling time of 23.3 hours, which is 7.9 hours greater than that of control cells i.e. 14.4 hours (Table 4.3.). Tobey and

Crissman, (1975) also showed CHO cells to have a 12 hour increase in population doubling time following mutagen administration. In contrast V79-379A cells showed only a very slight increase in population doubling time from 11.4 to 12.3 hours. Following adaptive pre-treatment, by Protocol 2, increased lag times for both CHO-K1 and V79-379A cells were found, and may be explained by the findings of Roberts and Ward, (1973) who suggested that increases of this parameter were due to a delay in cell cycle progression following mutagen pre-treatment. This manifests as an increased length of time taken for the cells to attach to the culture vessel before commencement of growth.

The decrease in cell growth of CHO-K1 cells, following adaptive pre-treatment, and to a much lesser extent V79-379A cells, may offer one explanation for the decreased mutation by effectively increasing the mutant expression period. However, this does not necessarily explain the enhancement of survival seen in each cell line since Lankas *et al.*, (1979) reported no difference of MNNG-induced cytotoxicity between slow and fast growing cells. Once again the possibility that pre-treatment alters the proportion of pre-existing resistant cells, in the overall population, is suggested. This would result in an apparent increase in cell survival on mutagen challenge. A slowed growth rate of the sensitive sub-population may account for this enrichment. To clarify this theory, and to establish the contribution of each sub-population to the overall observed effects, it was necessary to isolate each sub-population and assess the effects of adaptive pre-treatment on the growth, MNNG-induced cytotoxicity and mutation to Oua^R of each. Isolation of the individual populations was based on their differential

sensitivity to MNNG cytotoxicity. MNNG-resistant colonies were easily isolated by ring cloning of colonies that survived a high dose of MNNG (0.3 μgml^{-1}). The isolated cells were designated CHO-K1R and V79-379AR (5.3.1.). Isolating the sensitive sub-population was not as simple, since the only way to identify this sub-population was to kill it. However, a solution to this problem was found with the use of a replica plating technique. A common technique in bacterial work, this technique has been less easily applied to mammalian cell systems. Following the initial studies of Esko and Raetz, (1978), who used filter paper discs for replica plating, Raetz *et al.*, (1982) reported an efficient and rapid method of replica plating using polyester mesh discs. This method was used to isolate MNNG-sensitive colonies of CHO-K1 and V79-379A cells and essentially involved the production of high quality replicas of a population of each cell line, subjecting them to a low dose of MNNG, identifying the colony (or colonies) that are absent (by comparison with an untreated replica) and then isolating this colony from the corresponding position on a master plate (Plates 5.1. and 5.2.). Isolation from this replica was achieved by ring cloning. The isolated cells were designated CHO-K1S and V79-379AS (5.3.2.). It must be emphasised, however, that the sucessful production of replicas could only be achieved if the discs were extremely clean. Any trace of the cleaning chemicals, particularly the detergent used, resulted in partial or complete cell death.

Although resistant cells were isolated by their ability to survive a 0.3 μgml^{-1} dose of MNNG they were not completely resistant to the cytotoxic action of MNNG challenge. Thus, dose-survivor curves for both CHO-K1R and V79-379AR cells were

produced. These cells were found to be 5.2 and 5.7 fold more resistant to the cytotoxic action of MNNG when compared, on a D_{37} basis, with their respective parental cells (Table 5.1.). Plating efficiencies of untreated CHO-K1R and V79-379AR cells ranged from 75-90% which were similar to those found for the parental cells. Resistant cells isolated by other workers have shown between 3.3 and 40 fold higher resistances to challenge by the selecting mutagen, when compared to their parental lines (Friedman and Huberman, 1980; Goldmacher *et al.*, 1986; Goth-Goldstein and Hughes, 1987a; Ishida and Takahashi, 1987). CHO-K1R cells exhibit survival that is a simple exponential function of dose whereas V79-379AR cells have a bi-phasic curve suggesting that these cell retain an element of sensitive cells i.e. approximately 40% (Figs.5.1. and 5.2.). CHO-K1R and V79-379AR cells exhibited population doubling times of 26.1 and 24.4 hours which are 11.7 and 13 hours greater than their respective parental lines (Tables 4.2 and 5.2.). It may be noted, at this point, that the population doubling time, derived for CHO-K1R cells, is similar to that obtained for CHO-K1 cells following adaptive pre-treatment i.e. 23.3 hours (Table 4.3.).

Both CHO-K1R and V79-379AR cells exhibited MNNG-induced mutation frequencies to Oua^R that were lower, on a dose basis, than those of parental cells. This may be attributable to the slower growth rate observed in the resistant cells. When the results were analysed at equicytotoxic doses, however, these cells were seen to be as equally mutable as their respective parental lines. On a dose to dose and equicytotoxic basis, the induced-mutation frequencies for each resistant line were comparable with those obtained for their respective parental cell

lines after they had been subjected to adaptive pre-treatment. This again suggests an enrichment of the population with the resistant sub-population, followed adaptive pre-treatment of parental cells.

Adaptive pre-treatment of CHO-K1R and V79-379AR cells did not alter any of the above parameters except for increasing the cytotoxic resistance of V79-379AR cells by a factor of 2 (i.e. D_{37} values of 0.07 to 0.13 μgml^{-1}). The bi-phasic nature of the V79-379AR dose-response curve was changed to a simple exponential decrease in cell survival by the adaptive pre-treatment, a fact that implies the incomplete elimination of the sensitive element of a V79-379A population during selection of the resistant sub-population. Adaptive pre-treatment has then completed this selection process.

Goth-Goldstein and Hughes, (1987a) described an MNNG-resistant CHO-9 clone (CHO-9-C13) which had survived a 3 μgml^{-1} MNNG treatment. This was found to be 8-fold more resistant to MNNG challenge and had the same population doubling time as the parental CHO-9 cells. They also reported this clone to have a parental modal chromosome number of 21. Friedman and Huberman, (1980) isolated MNNG resistant V79 cells (VR-43) that had a 3.3 fold greater resistance to MNNG challenge than the parental V79 cells. VR-43 cells had a mean population doubling time of 16±2 hours and a modal chromosomal number of 22 chromosomes which were the same as the parental values for these cells. These authors showed that MNNG treatment of VR-43 cells resulted in mutation frequencies to both Oua^R and $6TG^R$ that were lower, on a dose basis, than the parental line. However, when analysed at equicytotoxic doses of MNNG the VR-43 cells were found to be more

mutable than the V79 cells i.e. at doses yielding 37% survivors the VR-43 cells gave 2-fold higher mutation frequencies than the V79 cells. Goldmacher *et al.*, (1986) found their isolated MNNG-resistant TK6 MT1 cell line to be hypermutable following MNNG challenge and have a doubling time approximately 5 hours greater than that of the parental line i.e. 21-24 hours compared to 17-18 hours.

The MNNG-sensitive cell clones CHO-K1S and V79-379AS exhibited cell killing identical to the parental lines up to a dose level of 0.05 $\mu g ml^{-1}$. Above this level the cells appear to be much more sensitive to MNNG than their respective parental lines (Figs.5.1. and 5.2.). Consequently a comparison between the D_{37} values of parental and sensitive cells does not reflect the increased sensitivity of this cell line. Sensitivity was selected for during the isolation procedure where the more resistant sub-population was excluded by ring cloning. The second exponential phase of the parental survivor curve (resistant sub-population) is absent when survivor curves for these cell lines are constructed.

The growth parameters of CHO-K1S and V79-379AS were comparable to their parental values, but dramatically changed following adaptive pre-treatment. The population doubling time of CHO-K1S cells is increased by 94.1 hours from 14.4 to 108.5 hours and that of V79-379AS cells by 53.7 hours from 14.7 to 68.4 hours (Figs.5.6. and 5.7.). This observation explains the lack of success during the cytotoxicity and mutation experiments involving pre-treatment of these cells. Colony growth was not observed for either CHO-K1S or V79-379AS following adaptive pre-treatment and after 11 days incubation the experiments were abandoned.

Assessment of the induction of Oua^R mutants by MNNG in both sensitive cell lines showed them to exhibit higher mutation frequencies for each mutagen dose but to be as equally mutable as their respective parental line on analysis at equicytotoxic doses. Kaina (1987), reported that the Chinese hamster ovary clone , W27.1, was hypersensitive to MNNG at each dose level, when compared to the parental CHO-9 cells. This clone was isolated by replica plating on filter paper discs.

Sister chromatid exchanges are considered to be a useful indicator of the mutagenicity of alkylating agents and since the assessment of SCE was previously used as a mutagenic end-point during adaptation investigations, analysis of SCE was employed in the present studies. The sensitive cell lines CHO-K1S and V79-379AS were not assayed for induced SCE because they exhibited such a huge increase in doubling time, following adaptive pre-treatment, that incubation of cells with BrdU, for two rounds of DNA replication would have unreasonably lengthened the experiment. One other reason for not using these cell lines was the lack of cytotoxicity data concerning adaptively pre-treated cells, on which the dose range for SCE induction could be based. However, the parental and resistant cell lines were examined for MNNG-induced SCE. One surprising feature observed at the outset of this work, was that whilst CHO-K1 and V79-379A cells had a modal chromosome number of 20±2, the resistant cell lines (CHO-K1R and V79-379AR) contained a polyploid genome with a chromosome number of 39±2. It is well estabilised that various chemical agents, including alkylating agents, can interfere with the processes that underlie the distribution of chromosomes in the daughter cells resulting in

either aneuploid or polyploid cells (Bonatti *et al.*, 1986; Rainaldi *et al.*, 1987; Tsutsui *et al.*, 1986). Rainaldi *et al.*, (1987) suggested that chemically induced aneuploidy in their V79/AP4 cells may have arisen either by non-disjunction at division or by a total block in the formation of a functional metaphase spindle.

A dose-dependent induction of SCE in both CHO-K1 and V79-379A cells by MNNG was demonstrated and is shown in Figs.7.1. and 7.2. These data are in good agreement with that reported by Perry and Evans, (1975) who examined the MNNG-induced SCE in CHO cells. The concentration range over which MNNG produced the responses seen in these Figures is also similar to that reported by Perry and Evans i.e. 0.004 μgml^{-1} to 0.2 μgml^{-1}. These workers reported control (vehicle) SCE levels of 0.61 SCE per chromosome, a figure similar to the values found for CHO-K1 (0.44 SCE per chromosome) and V79-379A (0.54 SCE per chromosome). At dose levels of 0.2 μgml^{-1} MNNG CHO-K1 and V79-379A cells exhibited 3.4 and 4.38 SCE per chromosome respectively compared to 5.31 SCE per chromosome for CHO cells (Perry and Evans, 1975) and 3.0 SCE per chromosome for V79 cells (Popescu *et al.*, 1979).

Although the resistant cells have double the chromosome number of their respective parental cells this is not reflected in the numbers of MNNG-induced SCE. At each MNNG dose level studied the induced SCE per chromosome (with the relevant background count subtracted) were lower in the MNNG resistant clones (CHO-K1R and V79-379AR) when compared to the respective parental lines (Figs.7.1., 7.2. and 7.3.). The extra chromosomes may confer some resistance to SCE formation resulting in proportionally fewer SCE. If one considers the induction of SCE per cell, there were a

greater number of exchanges in the resistant cells, at a low MNNG dose (0.1 μgml^{-1}), than the parental cells but the numbers were equal at the higher dose levels (0.1 and 0.2 μgml^{-1}). It is conceivable that a cell, resistant or not, has a maximum level of possible SCE and the rate at which these are accumulated may vary from one cell line to another, but ultimately results in the same number of SCE per cell for a particular cell line. Alternatively the equallisation of SCE at higher doses may be a failing of the SCE visualisation technique, allowing only a limited number of SCE to be detected in any one cell. Krepinsky, (1979), however, scored a maximum of 300 EMS-induced SCE per cell in Blooms syndrome lymphocytes, which have a genome of approximately 46 chromosomes. Since the MNNG-resistant cells have 39±2 chromosomes per cell, it seems unlikely that a limitation of the visualisation technique would account for the lower SCE frequencies observed between parental cells and the derived MNNG-resistant clones..

Adaptive pre-treatment of both CHO-K1 and V79-379A cells resulted in lower MNNG-reduced SCE frequencies but only at challenge doses of 0.2 and 0.3 μgml^{-1} MNNG. Background levels of SCE are considerably higher in pre-treated cells than in untreated control cells with values of 2.21 vs 0.44 SCE per chromosome for CHO-K1 and 1.87 vs 0.54 SCE per chromosome for V79-379A cells. This suggests that lesions (mutations) responsible for SCE induction have accumulated during the pre-treatment period and that only SCE above these background levels are induced by the challenge doses. Therefore Figures 7.4. and 7.5. show the effect of adaptive pre-treatment on both cell lines expressed as SCE per chromosome with the relevant background count subtracted.

These results are in agreement with those reported by

Samson and Schwartz, (1980) who reported a resistance to SCE induction following adaptive pre-treatment. These authors also reported a 50% increase in background SCE levels for pre-treated cells. It was noticed, but not quantified, that populations of both CHO-K1 and V79-379A cells contained a small proportion of polyploid cells, which probably represents the resistant sub-population in each instance. Adaptive pre-treatment of these parental cells resulted in much greater percentages of polyploid cells i.e. CHO-K1 = 49% and V79-379A = 34% (Tables 7.3 and 7.4.). The presence of a greater proportion of polyploid cells, which may equate to the resistant cells CHO-K1R and V79-379AR, may explain the reduced SCE levels following pre-treatment, since the polyploid CHO-K1R and V79-379AR cells were shown to have SCE levels lower than the respective parental cells, at each dose level studied (Figs.7.1. 7.2. and 7.3.).

CHO-K1R and V79-379AR cells show little difference between the MNNG-induced SCE frequencies of pre-treated and untreated cells (Fig.7.6.). The background levels of SCE also remain unchanged following pre-treatment.

To elucidate whether the observed responses were due to pre-treatment induction of O^6MGMT or pre-treatment selection of pre-existing resistant cells, it was necessary to determine the presence of this enzyme in both CHO-K1 and V79-379A cells. This was performed by assessing the ability of cell-free extracts of each cell line to de-methylate an alkylated exogenous DNA substrate using a HPLC technique to separate the alkylated bases following acid hydrolysis of the substrate. To validate the de-methylation assay a cell line was used which had previously

been shown to possess the O^6MGMT enzyme i.e. the mouse C3H $10T^1/_2$ line (Grisham and Smith, 1984). Details are presented in Chapter 6. This cell line de-methylated alkylated DNA at a rate of 23.3 pmoles O^6MG/mg protein/hour (Table 6.4.) (6.5.1.). Since a great deal of de-methylation work has been carried out using radiolabelled substrates (Domoradzki *et al.*, 1985; Foote and Mitra, 1984; Ishida and Takahashi, 1987; Lefebvre and Laval, 1986; Yagi *et al.*, 1984), it is difficult to directly compare the data presented in Chapter 6 with the published data. The de-methylation of O^6MG, in an alkylated DNA substrate has been expressed as relative counts per minute (cpm) removed per mg protein, pmols removed per mg protein and fmols removed per mg protein. Foote and Mitra, (1984), using a 8-^{3}H-labelled poly(dC, dG, m^6dG) polymer, reported Hela CCL3 cells to de-methylate the substrate to the order of 0.4 pmol O^6MG de-methylated per mg protein. Ishida and Takahashi, (1987), also using the HeLa cell line, reported 1.25 pmol de-methylated per mg protein. Both were estimated after a 4 hour incubation of cell extract with substrate. Lefebvre and Laval, (1986) reported 0.091 pmol O^6MG de-methylated per mg protein for extracts of rat H_4 cells, whereas Domoradzki *et al.*, (1985) reported 0.24 pmol O^6MG de-methylated per mg protein for extracts of normal human fibroblasts.

Smith *et al.*, (1981) incubated C3H $10T^1/_2$ cells with ^{14}C-MNNG and, at specific times over a 34 hour period, harvested cells and estimated the amount of O^6MG present in the cells' DNA by chromatographic separation of the individual bases on a Sephadex G-10 column. The results were expressed as the frequency of lesions, calculated as adducts per 10^6 guanine molecules, in the DNA recovered. They reported a 49% reduction of O^6MG content

in these cells over a 34 hour period following MNNG administration. The results for C3H $10T^1/_2$ presented in Table 6.4. show a 66% reduction in the content of O^6MG over a period of 1 hour. Although the de-mehtylation rate is more rapid than that reported by Smith *et al.*, (1981), this assay system was nevertheless considered valid for the de-methylation studies undertaken for this thesis.

Using this assay technique it was shown that cell-free extracts of CHO-K1, CHO-K1R, V79-379A and V79-379AR cell lines did not de-methylate alkylated DNA, above the control values, with or without adaptive pre-treatment (Tables 6.6. and 6.7.). The partial de-methylating ability of each cell line can be attributed to spontaneous substrate de-methylation since a control, which used BSA instead of a cell-free extract, also showed a small degree of de-methylation ability i.e. 2.7 pmoles O^6MG de-methylated/ mg protein/hour (Table 6.5.). The conclusion to be drawn from this series of experiments is that the hamster lines studied are devoid of demonstrable O^6MGMT enzyme activity and that the cells are either totally deficient of the enzyme or the enzyme is inactive in these cell lines.

Two groups, Waldstein *et al.*, (1982c) and Lefebvre and Laval, (1986) have reported the presence of O^6MGMT in the CHO cell line, but only at low, constitutive levels. However induction of this enzyme following mutagen pre-treatment was not observed by either group. Waldstein *et al.*, (1982a), however, reported a 2-3 fold enhancement in O^6MGMT activity of HeLa CCL2 cells, following an adaptive pre-treatment protocol that involved a 6-hourly administration of 25 $ngml^{-1}$ MNNG over a 24 hour period. In contrast, Foote and Mitra, (1984) failed to observe induction of

this enzyme in either rat or CHO cells, using essentially the same pre-treatment protocol.

The existence of O^6MGMT in Chinese hamster lines has been disputed by a number of authors (Foote *et al.*, 1983; Foote and Mitra, 1984; Goth-Goldstein, 1980; Harris *et al.*, 1983; Yarosh *et al.*, 1984). One group i.e. Harris *et al.*, (1983) pointed out that the concentration of protein used in Waldsteins' assay on crude extracts was too low, and that substantial differences in enzyme contents might be masked by this fact. Under such experimental conditions spontaneous depurination may be mistaken for actual O^6MG removal by these cells.

It may be concluded, from the evidence reported above and from previous reports, that Chinese hamster cell lines do not possess any demonstrable O^6MGMT activity and that any adaptive-like effects observed, are almost certainly due to mechanisms other than O^6MGMT induction.

8.2. Concluding discussion.

The results of the present investigation have shown that CHO-K1 and V79-379A cells, following adaptive mutagen pre-treatment by a protocol that incorporates a chronic dose schedule, exhibit a bacterial-like adaptive response. However, on close scrutiny it was discovered that the reductions in MNNG challenge-induced cytotoxicity, mutation to Oua^R and SCE frequency, that were observed after MNNG pre-treatment, were not a true adaptive response but were a protocol-mediated effect. It was found that pre-treatment by Protocol 2, increased the proportion of the more resistant sub-population via a growth suppression of the more sensitive sub-population, possibly combined with the induction of polyploid cells that were shown to be mutagen resistant.

Total absence, or inactivity, of the enzyme O^6MGMT,in these cells suggests that the observed cytotoxicity, mutation and SCE responses are brought about by a mechanism other than that of increased enzymatic repair of alkylated DNA. The increased survival of pre-treated and then mutagen challenged cells, therefore, was attributed to the increased proportion of resistant cells. The observed reduction in mutation frequencies, in the pre-treated cells, was considered to be due to the lower number of mutants produced by the slower growing cells, i.e. the inability of these cells to reach the optimum mutant expression time.

The two questions asked at the outset of this investigation i.e. (1) are the discrepancies in the data, previously found by other workers, that have been reported, due to the different protocols used and (2) are the contrasting results due to the

different cell types used, can be answered in the light of the present findings. Protocol structure seems to be very important when designing adaptation experiments since protocol 2 evoked an apparent response in both CHO-K1 and V79-379A cells, whereas Protocol 1 did so only in the V79-379A cell line. Using Protocol 2 throughout the subsequent adaptation studies it was apparent that there were few inter-cell line differences when the effects of adaptive pre-treatment were assayed by a number of different biological end-points. It would seem therefore, that in order to make valid comparisons of the adaptive response between cell lines and between laboratories using different cell lines, a standard pre-treatment protocol must first be agreed upon if any meaningful work is to ensue.

8.3. Suggestions for Future Work.

1) To confirm the theory that the reduced mutation, on a dose basis, observed in adapted CHO-K1 and V79-379A cells, is attributable to the slowed growth of these cells, compared to parental growth rates, an experiment should be performed in which the optimum mutant expression time of pre-treated cells is determined. This would elucidate whether, given the correct expression time, these cells could demonstrate the same number of mutants as the control cells.

2) Reduced mutation in pre-treated cells may arise by the actvity of an enzyme other than O^6MGMT. Saffhill and Fox, (1980) reported that V79 cells were competent at de-methylating O^4-methylthymidine within their DNA and suggested that since these cells do not possess O^6MGMT that a different enzyme may be responsible for the observed reduction of mutation. The existence of this enzyme in CHO-K1 and V79-379A cells cannot be ruled out since the activity was not assayed. Each cell line could be cultured in the presence of O^4-methyl-[6-^{3}H]-thymidine allowing incorporation into the cellular DNA, and at various time intervals following this procedure, the de-methylation of O^4-methylthymine could be shown by assessing the total amount of alkylations present in the DNA.

3) One other useful study would be to determine whether an enzyme activity similar to the 3-MAG activity found in bacteria, exists in these cell lines, and if so can it be induced by adaptive pre-treatment. The assay system used to determine the de-methylation of O^6MG in alkylated DNA was inadequate for this purpose since 3MG could not be differentiated from an acid-hydrolysed mixture of alkylated bases (not shown), even

though separation was possible when a standard mixture of eight methylated bases was analysed.

4) One interesting result to emerge during the course of these studies was the fact that certain CHO-K1 and V79-379A cells have an inherent resistance to MNNG cytotoxicity. The appearance of resistant cells is a worrying factor when one considers cancer chemotherapy involving alkylating agents, since a hyper-resistant tumour, arising by either enzymatic or sensitive cell-suppression mechanisms, would be resistant to further chemotherapy by the same agent. It would be useful to elucidate the mechanism of MNNG resistance by studying the cell lines CHO-K1R and V79-379AR. The level of intercellular thiol has been suggested as the reason for the resistance and if true, could be relevant to the cytotoxic efficiency of drugs such as BCNU and CCNU, which require thiols to produce the active carbonium ion intermediate. Since cancer chemotherapy regimes are continued for long periods the likelyhood of the appearance of resistant cells is great. One possible mechanism of resistance, could be the reduced uptake of mutagen into the resistant cells, by a depleted membrane transport system, for instance. Studies into the relative uptake of ^{14}C-MNNG could be performed and the efficiency of CHO-K1R and V79-379AR cells could be compared with the uptake observed in the parental CHO-K1 and V79-379A cells. Of greater relevance would be to study the relative uptake rates of anti-neoplastic drugs that are routinely used in cancer chemotherapy and to evaluate their cytotoxic potency to normal and resistant cells.

In conclusion it is suggested that future studies into the adaptive response should be performed using human cell lines, since the results of *in vitro* experiments will ultimately need to

be extrapolated back to the human situation.

REFERENCES

Ahmed, Z. and Laval, J. (1984). Biochem. Biophys. Res. Comm.. 120. 1.

Ahmed, F.E., Lewis, N.J. and Hart, R.W. (1977). Chem-Biol. Interact. 19. 369.

Althouse, R. (1979). Br. Med. J. 1. 1630.

Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. (1973). Proc. Natl. Acad. Sci. USA. 70. 2281.

Arlett, C.F. (1977). In: D.Scott, B.A. Bridges and F.H. Sobels (Eds.). Progress in Genetic Toxicology, Elsevier, Amsterdam. p141.

Arlett, C.F. and Lehmann, A.R. (1978). Ann. Rev. Genet. 12. 95.

Arlett, C.F., Turnbull, D., Harcourt, S.A., Lehmann, A.R. and Colella, C.M. (1975). Mutat. Res. 33. 621.

Auerbach, C. (1976). Mutation Research: Problems, Results and Perspectives, Chapman and Hall, London.

Auerbach, C. and Robson, J.M. (1946). Proc. Roy. Soc. Edinburgh. 62. 271.

Baker, R.M., Brunette, D.M., Mankovitz, R., Thompson, L.H., Whitmore, G.F., Siminovitch, L. and Till, J.E. (1974). Cell. 1. 9.

Baker, R.M., van Voorhis, W.C. and Spencer, L.A. (1979). Proc. Natl. Acad. Sci. USA. 76. 5249.

Balsiger, R.W. and Montgomery, J.A. (1960). J. Organic Chem. 25. 1573.

Bambourschke, S., O'Connor, P.J., Kleihues, P. and Maru, G.B. (1983). Cancer Res. 43. 1306.

Barranco, S.C. and Humphrey, R.M. (1971). Mutat. Res. 11. 421.

Bedell, M.A., Lewis, J.G., Billings, K.L. and Swenberg, S.A. (1982). Cancer Res. 42. 3079.

Bell, J. (1876). Edinburgh Med. J. 22. 135.

Beranek, D.T., Heflich, R.H., Kodell, R.L., Morris, S.M. and Casciano, D.A. (1983). Mutat. Res. 110. 171.

Bignami, M., Terlizzese, M., Zijno, A., Calcagnile, A., Frosina, G., Abbondandolo, A. and Dogliotti, E. (1987). Carcinogenesis. 8. 1417.

Bodell, W.J., Aida, T., Berger, M.S. and Rosenblum, M.L. (1986). Carcinogenesis. 7. 879.

Bogden, J.M., Eastman, A. and Bresnick, E. (1981). Nucleic Acid Res. 9. 3089.

Boiteux, S. and Laval, F. (1985). Carcinogenesis. 6. 805.

Bonatti, S., de Ferrari, M., Pisano, V. and Abbondandolo, A. (1986). Carcinogenesis. 7. 99.

Boutiboues, P. and Auffrey, Y. (1987). Mutat. Res. 190. 83.

Bradford, M.M. (1976). Analyt. Biochem. 72. 248.

Bradley, M.O., Bhuyan, B., Francis, M.C., Langenbach, R., Peterson, A. and Huberman, E. (1981). Mutat. Res. 87. 81.

Brent, T.P. (1979). Biochemistry. 18. 911.

Brent, T.P. (1986). Cancer Res. 46. 2320.

Butlin, H.T. (1892). Brit. Med J. 2. 1.

Cairns, J., Robins, P., Sedgwick, B. and Talmud, P. (1981). Prog. Nucleic Acid Res. and Molec. Biol. 26. 237.

Carrano, A.V., Thompson, L.H., Lindl, P.A. and Minkler, J.L. (1978). Nature. 271. 551.

Carver, J.H., Hatch, F.T. and Branscomb, E.W. (1979). Nature. 279. 154.

Caskey, C.T. and Kruh, G.D. (1979). Cell. 16. 1.

Cathcart, R. and Goldthwait, D.A. (1981). Biochemistry. 20. 273.

Chu, Y.H., Craig, A.W. and O'Connor, P.J. (1981). J. Cancer. 43. 850.

Chu, E.H.Y. and Malling, H.V. (1968). Proc. Natl. Acad. Sci. USA. 61. 1306.

Clemmensen, J. (1951). J. Natl. Cancer Inst. 12. 1.

Cleaver, J.E. (1968). Nature. 218. 652.

Cole, J. and Arlett, C.F. (1976). Mutat. Res. 34. 507.

Cooper, D.P., O'Connor, P,J. and Margison, G.P. (1982). Cancer Res. 42. 4203.

Craddock, V.M., Henderson, A.R. and Gash, S. (1982). Biochem. Biophys. Res. Comm. 107. 546.

Davies, P.J. and Parry, J. (1974). Genet. Res. 24. 311.

Day, R.S., Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S.A., Lubiniecki, A.S., Girardi, A.J., Galloway, S.M. and Bynum, G.D. (1980). Nature. 288. 724.

Demple, B., Karran, P. and Lindahl, T. (1983). In: E.C. Friedberg and P.C. Hanawalt (Eds.). DNA repair, Vol.2, Dekker, New York, p41.

Dewdney, R.S. (1982). Ph.D. thesis, University of Bath.

Dolan, M.E., Morimoto, K. and Pegg, A.E. (1985). Cancer Res. 45. 6413.

Domoradzki, J., Pegg, A.E., Dolan, M.E., Maher, V.M. and McCormick, J.J. (1984). Carcinogenesis. 5. 1641.

Domoradzki, J., Pegg, A.E., Dolan, M.E., Maher, V.M. and McCormick, J.J. (1985). Carcinogenesis. 6. 1823.

Drahovsky, D. and Wacker, A. (1975). Eur. J. Cancer. 11. 517.

Drake, J.W. and Baltz, R.H. (1986). Ann. Rev. Biochem. 45. 11.

Dulbecco, R. and Vogt, M. (1954). J. Exptl. Med. 99. 167.

Durrant, L.G., Margison, G.P. and Boyle, J.M. (1981). Carcinogenesis. 2. 55.

Elkind, M.M. and Sutton, R. (1959). Nature. 184. 1293.

Englese, L.D., Floot, B.G.J., Merkveld, G.J. and Tates, A.D. (1986). 7. 1941.

Esko, J.D. and Raetz, C.R.H. (1978). Proc. Natl. Acad. Sci. USA. 75. 1190.

Evans, H.J. (1977). In: de la Chapelle, A. and Sorsa, M. (Eds.). Chromosomes Today, Vol.6, Elsevier, Amsterdam, p315.

Eveson, G. and Seeberg, E. (1982). Nature. 296. 773.

Fishbein, L. (1979). Potential Industrial Carcinogens and Mutagens. Elsevier, Amsterdam.

Foote, R.S. and Mitra, S. (1984). Carcinogenesis. 5. 277.

Foote, R.S., Pal, B.C. and Mitra, S. (1983). Mutat. Res. 119. 221.

Ford, D.K. and Yerganian, G. (1958). J. Natl. Cancer Inst. 21. 393.

Fox, M. and Carlton, C.M. (1984). Carcinogenesis. 5. 1531.

Fox, M., McMillan, S., Durrant, L. and Boyle, J.M. (1982a). Mutat. Res. 95. 339.

Fox, M., Sultani-Makzoumi, C.M. and Boyle, J.M. (1982b). Biochimie. 64. 687.

Freshney, R.I. (1983). Culture of animal cells: A manual of basic Techniques, Alan R. Liss, Inc., New York.

Friedberg, E.C. (1985). DNA repair, W.H. Freeman and Co., New York.

Friedman, J. and Huberman, E. (1980). Proc. Natl. Acad. Sci. USA. 77. 6072.

Frosina, G. and Abbondandolo, A. (1985). Mutat. Res. 154. 85.

Frosina, G., Bonatti, S. and Abbondandolo, A. (1984). Mutat. Res. 128. 243.

Frosina, G. and Laval, F. (1987). Carcinogenesis. 8. 91.

Gerson, S.L., Trey, J.E., Miller, K. and Beger, N.A. (1986). Carcinogenesis. 7. 745.

Goldmacher, V.S., Cuzick, R.A. and Thilly, W.G. (1986). J. Biol. Chem. 261. 12462.

Gombar, C.T., Katz, E.J., Magee, P.N. and Sirover, M.A. (1981). Carcinogenesis. 2. 595.

Goth-Goldstein, R. (1980). Cancer Res. 40. 2323.

Goth-Goldstein, R. (1987). Carcinogenesis. 8. 1449.

Goth-Goldstein, R. and Hughes, M. (1987a). Mutat. Res. 184. 139.

Goth-Goldstein, R. and Hughes, M. (1987b). Mutat. Res. 177. 267.

Green, M.O. and Greenberg, J. (1960). Cancer Res. 20. 1166.

Greenberg, D.S., Grisham, J.W., Bell, W.N., Baker, M.S. and Kaufman, D.G. (1978). In vitro. 14. 516.

Grisham, J.W. and Smith, G.J. (1984). Pharmacol. Rev. 36. 151S.

Gupta, S. and Siminovitch, L. (1980). Mutat. Res. 69. 113.

Hadden, C.T., Foote, R.S. and Mitra, S. (1983). J. Bacteriol. 153. 756.

Hall, J., Bresil, H. and Montesano, R. (1985). Carcinogenesis. 6. 238.

Ham, R.G. and Puck, T.T. (1963). In: Colowick, S.P. and Kaplan, N.O. (Eds.). Methods in Enzymology, Vol.5., Academic Press, New York, p90.

Harris, A.L., Karran, P. and Lindahl, T. (1983). Cancer Res. 43. 3247.

Hart, R.W., Hall, K.Y. and Daniel, B.F. (1978). Photochem. Photobiol. 28. 131.

Hecht, S.S., Chen, C.B., Hirota, N., Ornaf, R.M., Tso, T.C. and Hoffman, D. (1978). J. Natl. Cancer Inst. 60. 819.

Hecht, S.S., Chen, C.B., Ohmori, T. and Hoffman, D. (1980). Cancer Res. 40. 298.

Heflich, R.H., Beranek, D.T., Kodell, R.L. and Morris, S.M. (1982). Mutat. Res. 106. 147.

Heidelberger, C. (1975). Ann. Rev. Biochem. 44. 79.

Hsie, A.W., Brimer, P.A., Mitchell, T.J. and Goslee, D.G. (1975). Somat. Cell. Genet. 1. 247.

Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, D.F., O'Neill, J.P. and Whitfield, B.L. (1981). Mutat. Res. 86. 193.

Hyde, D.A., Acton, E. and Skinner, W.A. (1962). J. Med. Pharm. Chem. 5. 1.

Ishida, R. and Takahashi, T. (1987). Carcinogenesis. 8. 1109.

Ishizaki, K., Tsujimura, T., Fujio, C., Yangpei, Z., Yawata, H., Nakabeppu, Y., Sekiguchi, M. and Ikenaga, M. (1987). Mutat. Res. 184. 121.

Jeggo, P., Defais, M., Samson, L. and Schendel, P. (1978). Mol. Gen. Genet. 162. 299.

Jensen, D.E. and Magee, P.N. (1981). Cancer Res. 41. 230.

Kaina, B. (1982). Mutat. Res. 93. 195.

Kaina, B. (1983a). Mutat. Res. 111. 341.

Kaina, B. (1983b). Carcinogenesis. 4. 1437.

Kaina, B. (1987). Carcinogenesis. 8. 1935.

Kao, F.T. and Puck, T.T. (1968). Proc. Natl. Acad. Sci. USA. 60. 1275.

Kao, F.T. and Puck, T.T. (1969). J. Cell. Physiol. 74. 245.

Kao, F.T. and Puck. T.T. (1974). J. Meth. Cell. Biol. 8. 23.

Karran, P., Helmgren, T. and Lindahl, T. (1982). Nature. 296. 770.

Karran, P., Lindahl, T. and Griffin, B. (1979). Nature. 280. 76.

Kennaway, E.L. (1955). Brit. Med J. 11. 749.

Krepinsky, A.B., Heddle, J.A. and German, J. (1979). Human Genet. 50. 151.

Krokan, H., Haugan, A., Myrnes, B. and Guddal, P.H. (1983). Carcinogenesis. 4. 1559.

Lankas, G.R. (1979). Mutat. Res. 60. 189.

Lankas, G.R.,Baxter, C.S. and Christian, R.T. (1977). Mutat. Res. 45. 153.

Latt, S.A. (1975). Proc. Natl. Acad. Sci. USA. 70. 3395.

Laval, F. (1986). IARC. Sci. Publ. 70. 321.

Laval, F. and Laval, J. (1984). Proc. Natl. Acad. Sci. USA. 81. 1062.

Lawley, P.D. (1974). Mutat. Res. 23. 283.

Lawley, P.D. and Thatcher, C.J. (1970). Biochem. J. 116. 693.

Lefebvre, P. and Laval, F. (1986). Cancer Res. 46. 5071.

Lehmann, A.R. and Mayne, L. (1981). In: Chromosome Damage and Repair, Seeberg, E. and Kleppe, K. (Eds.), Plenum, New York., p367.

Lever, J.E. and Seegmuller, J.E. (1976). J. Cell. Physiol. 88. 343.

Lijinsky, W. (1980). Oncology. 37. 223.

Lijinsky, W. (1984). In: Genotoxicity of N-nitroso compounds. Rao, W. and Epler, J.L. (Eds.), Plenum, New York.

Lindahl, T., Karran, P., Demple, B., Sedgwick, B. and Harris, A. (1982). Biochimie. 64. 581.

Lindahl, T., Sedgwick, B., Demple, B. and Karran, P. (1983). In: Cellular Responses to DNA Damage, Alan R. Liss, Inc., p241.

Lindamood, C., Bedell, M.A., Billings, K.C., Dyroff, M.C. and Swenberg, J.A. (1984). Cancer Res. 44. 196.

Magee, P.N. and Barnes, I.M. (1956). Brit. J. Cancer. 10. 114.

Malaveille, C., Kuroki,, T., Sims, P., Grover, P.L. and Bartch, H. (1977). Mutat. Res. 44. 313.

Margison, G.P. (1982). Chem-Biol Interact. 38. 189.

Margison, G.P., Butler, J. and Hoey, B. (1985). Carcinogenesis. 6. 1699.

Margison, G.P., Cooper, D.P., Smith, R.A. Montesano, R., Bresil, H., Planche-Martel, G. and O'Connor, P.J. (1984). Folia Biologica. Special Edition. 132.

Margison, G.P., Swindell, J.A. Ockey, C.H. and Craig, A.W. (1980). Carcinogenesis. 1. 91.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Proc. Natl. Acad. Sci. USA. 72. 5135.

McCarthy, T.V., Karran, P. and Lindahl, T. (1984). Embo J. 3. 545.

McKay, A.F. (1948). J. Am. Chem. Soc. 71. 1411.

Mergens, W.J., Vane, S.M., Tannenbaum, S.R., Green, L. and Skipper, P. (1979). J. Pharm. Sci. 68. 827.

Meyn, R.E. and Murray, D. (1984). Pharmac. Therapeutics. 24. 147.

Montesano, R., Bresil, H., Drevon, C. and Piccoli, C. (1982). Biochimie. 64. 591.

Montgomery, J.A., McCaleb, G.S. and Kirk, M.C. (1975). J. Med. Chem. 18. 568.

Morgan, W.F. and Crossen, P.E. (1977). Cell. 8. 573.

Morris, S.M., Heflich, R.H., Beranek, D.T. and Beland, F.A. (1984). Basic Life Sci. 291. 353.

Munson, R.J. and Goodhead, D.T. (1977). Mutat. Res. 42. 145.

Murray, V. (1987). Mutat. Res. 190. 267.

Natarajan, A.T., Simons, J.W., Vogen, E.W. and van Zeeland, A.A. (1984). Mutat. Res. 128. 31.

Newbold, R.F., Warren, W. and Medcalf, A. (1980). Nature. 283. 596.

O'Connor, P.J. and Margison, G.P. (1981). In: Seeberg, E. and Kleppe, K (Eds.). Chromosome Damage and Repair, Plenum, New York, p233.

O'Neill, J.P., Brimer, P.A., Machanoff, R., Hirsch, R. and Hsie, A.W. (1977). Mutat. Res. 45. 91.

O'Neill, J.P., Schenley, R.L. and Hsie, A.W. (1979). Mutat. Res. 63. 381.

Olsson, M. and Lindahl, T. (1980). J. Biol. Chem. 255. 10569.

Paterson, M.C. (1979). Ann Rev. Genet. 13. 291.

Paul, J. (1975). Cell and Tissue Culture, 5th Ed., Churchill Livingstone, Edinburgh.

Pegg, A.E., Perry, W. and Bennett, R.A. (1981). Biochem. J. 197. 195.

Pegg, A.E., Roberfroid, M., von Bahr, C., Foote, R.S., Mitra, S., Bresil, A., Likachev, A and Montesano, R. (1982). Proc. Natl. Acad. Sci. USA. 79. 5162.

Pegg, A.E. and Weist, L. (1983). Cancer Res. 43. 972.

Pegg, A.E., Weist, L., Foote, R.S., Mitra, S. and Perry, W. (1983). J. Biol. Chem. 258. 2327.

Pott, P. (1775). Printed in Natl. Cancer Inst. Monograph. 10. 7. (1963).

Perry, P. and Evans, H.J. (1975). Nature. 258. 121.

Perry, P. and Wolff, S. (1974). Nature. 251. 156.

Peterson, A.R., Peterson, H. and Heidelberger, C. (1979). Cancer. Res. 39. 131.

Popescu, S.S., Amsbaugh, P. and Dipaulo, J.A. (1979) Mutat. Res. 60. 313.

Preussman, R. and Stewart, B.W. (1984). In: Chemical Carcinogens. Searle, C.E. (Ed.). Am. Chem. Soc. Monograph, Vol.182., p643.

Raetz, C.R.H., Wermuth, M.M., McIntyre, T.M., Esko, J.D. and Wing, D. (1982). Proc. Natl. Acad. Sci. 79. 3223.

Rainaldi, G., Flori, L. Colella, C.M., Mariani, T., Piras, A., Sirica, S. and Simili, M. (1987). Mutat. Res. 177. 255.

Ratko, T.A. and Pezzuto, J.M. (1985). J. Chrom. 324. 484.

Reznikoff, C.A., Brankow, D.W. and Heidelberger, C. (1973). Cancer Res. 33. 3231.

Rhodes, J.W. and Johnson, D.E. (1972). Nature. 236. 307.

Riazzudin, S. and Lindahl, T. (1978). Biochemistry. 17. 2110.

Roberts, J.J. (1978). Adv. Radiat. Biol. 7. 211.

Roberts, J.J., Pascoe, J.M., Plant, J.E., Sturrock, J.E. and Crathorn, A.R. (1971). Chem-Biol. Interact. 2. 29.

Roberts, J.J. and Ward, K.N. (1973). Chem-Biol. Interact. 7. 241.

Robins, P. and Cairns, J. (1979). Nature. 280. 74.

Saffirman, C., Ionescu, A., Vioculet, N. and Niculescu, I. (1984). Rev. Roum. Biochem. 21. 199.

Saffhill, R. and Fox, M. (1980) Carcinogenesis. 1. 487.

Samson, L. and Cairns, J. (1977). Nature. 267. 281.

Samson, L. and Linn, S. (1987). Carcinogenesis. 8. 277.

Samson, L. and Schwartz, J.L. (1980). Nature. 287. 861.

Scanlan, R.A. (1975). CRC Critical Reviews in Food Technology. 5. 357.

Schendel, P. (1981). CRC Critical Reviews in Toxicol. 8. 311.

Schendel, P. and Robins, P.E. (1978). Proc. Natl. Acad. Sci. USA. 75. 6017.

Schwartz, J.L. (1986). Carcinogenesis. 7. 159.

Schwartz, J.L. and Samson, L. (1983). Mutat. Res. 119. 393.

Scudiero, D.A., Meyer, S.A., Clatterbuck, B.E., Matern, M.R., Ziolkowski, C.H.J. and Day. R.S. (1984). Cancer Res. 44. 961.

Sedgwick, B. (1982). J. Bacteriol. 150. 984.

Selkirk, J.K., MacCleod, M.C., Moore, C.J. and Dearstone, K. (1982). In: Mechanisms of Chemical Carcinogenesis. Harris, C.C. and Cerutti, P.A. (Eds.). Alan R. Liss, Inc., New York., p331.

Setlow, R.B. (1978). Nature. 271. 713.

Setlow, R.B., Waldstein, E.A., Cao, E.H., Muller, M.E. and Cronkite, E.P. (1982). Proc. Am. Assoc. Cancer Res. 23. 13.

Sieber, S.M. and Adamson, R.H. (1975). Adv. Cancer. Res. 22. 57.

Singer, B. (1979). J. Natl. Cancer Inst. 62. 1329.

Singer, B. and Kusmierek, J.T. (1982). Ann. Rev. Biochem. 52. 655.

Sklar, R.M. and Strauss, B.S. (1983). 43. 3316.

Smith, G.J., Grisham, J.W. and Kaufman, D.G. (1981). Cancer Res. 41. 1373.

Smith, R.A. and Margison, G.P. (1981). Br. J. Cancer. 44. 273.

Tannenbaum, S.R., Archer. M.C., Wishnok, J.S., Correa, P., Cuello, C. and Haenszel, W. (1977). In: Origins of Human Cancer. Hiatt, H.H., Watson, J.D. and Winston, J.A. (Eds.). Coldspring Harbour Conferences on Cell Proliferation. 4. p1609.

Taylor, J.H. (1958). Genetics. 43. 515.

Taylor, H.W. and Lijinsky, W. (1975). Cancer Res. 35. 812.

Thompson, L.H. (1978). Meth. in Enzymology. 58. 308.

Thompson, L.E., Dillehey, L.E., Carrano, A.A., Mazrimas. J.A., Mooney, C.L.and Minkler, J.L. (1982). Mutat. Res. 95. 427.

Tobey, R.A. and Crissman, H.A. (1975). Cancer Res. 35. 460.

Tsutsui, T., Suzuki, N., Maizumi, H. and Barrett, J.C. (1986). Carcinogenesis. 7. 131.

U.S. National Formulary. (1975). Vol. 14.

Valencia, R., Cong., N-G. and Bertaux, O. (1985). J. Chrom. 325. 207.

Venitt, S. and Parry, J.M. (Eds.) (1984). Mutagenicity Testing - A Practical Approach, IRL, Oxford.

Vistica, D.T. (1979). Biochim. et Biophys. Acta. 550. 309.

Von Volkemann, R. (1875). Beitrage zur Chirurgie (Leipzig).

Waldstein, E.A., Cao, E.H., Bender, M.A. and Setlow, R.B. (1982a). Mutat. Res. 95. 405.

Waldstein, E.A., Cao, E.H. and Setlow, R.B. (1982b). Proc. Natl. Acad. Sci. USA. 79. 5117.

Waldstein, E.A., Cao, E.H. and Setlow, R.B. (1982c). Nucleic Acid Res. 10. 4575.

Wang, R.J., Stoien, J.D. and Landa, F. (1974). Nature. 247. 43.

Warren, W. (1984). In: Mutagenicity Testing - A Practical Approach. Venitt, S. and Parry, J.M. (Eds.). IRL, Oxford, p25.

Watson, J.D. (1976). Molecular Biology of the Gene. 3rd Ed. Benjamin Inc., Menlo Park.

Weinstein, I.B., Jeffrey, A.M., Jenette, K.W., Blobstein, S.H., Harvey, R.G., Harris, C., Autrup, H. and Nakanishi, K. (1976). Science. 193. 592.

Wheeler, G.P. and Bowden, B.J. (1972) Biochem. Pharm. 21. 265.

White, G.R.M., Ockey, C.H., Brennand, J. and Margison, G.P. (1986). Carcinogenesis. 7. 2077.

Williams, D.H. and Fleming, I. (1980). Spectroscopic Methods in Organic Chemistry. 3rd Ed., McGraw Hill, UK.

Wilson, S.A. (1985). Ph.D. Thesis, University of Bath.

Wolff, S., Bodycote, J., Thomas, G.H. and Cleaver, J.E. (1975). Genetics. 81. 349.

Yagi, T., Yarosh, D.R. and Day, R. (1984). Carcinogenesis. 5. 593.

Yarosh, D.B., Foote, R.S., Mitra, S. and Day, R. (1983). Carcinogenesis. 4. 199.

Yarosh, D.B., Rice, M., Day. R., Foote, R.S. and Mitra, S. (1984). Mutat. Res. 131. 27.

Yarosh, D.B., Fornace, A.J. and Day, R.S. (1985). Carcinogenesis. 6. 949.

Yarosh, D.B., Hurst-Calderone, S., Babich, M.A. and Day, R. (1986). Cancer Res. 46. 1663.

Zwanenburg, T.S.B. (1983). Mutat Res. 120. 151.